Mechanisms underlying disease transmission between spatially separated animals

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Voor Pa

Abstract

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Transmission of infections between spatially separated hosts is a common problem, not only during major outbreaks of livestock diseases, but also in many other settings such as the transmission of infectious diseases between plants and crops or in healthcare settings. During the last major epidemics of livestock diseases in the Netherlands and abroad, disease transmission events occurred despite movement bans and other (bio-)security measures, implying that indirect transmission plays a major role. A better understanding of indirect transmission is necessary to put in place evidence based bio-security measures against neighbourhood (indirect) transmission. To gain more insight in the mechanisms underlying indirect transmission a series of experimental studies combined with mathematical modelling were conducted of which the results are presented in this thesis. First the effect of acidification of drinking water on the transmission parameters of direct and indirect transmission of *Campylobacter jejuni* (*C. jejuni*) between broilers was studied. It was shown that acidified drinking water has an effect on indirect transmission but not on direct transmission of *C. jejuni* between broilers. The sender and receiver sub-process of indirect transmission was then studied in more detail and it was shown that a significant negative interaction effect between acidification of the sender and receiver sub-processes exists, indicating that there is no additional effect of acidification of the drinking water on both sides of the transmission process compared to acidified drinking water only on one side. To study the transport of the pathogen in the environment in more detail, a series of indirect transmission experiments was carried out and a model framework was developed to study indirect transmission between spatially separated hosts. These studies showed that indirect transmission of *C. jejuni* between broilers is best described by a multistage environmental route from sending to receiving animal, suggesting that indirect transmission occurs through progressive (but slow) contamination of the environment surrounding the source. Indirect transmission experiments where repeated with both *C. jejuni* and *Escherichia coli* and the results showed that for *C. jejuni* it takes much longer for the first effective (viable) bacterium to cross the small distance of approximately 75 cm than it does for *Escherichia coli*. A new modelling approach to study indirect transmission was developed guided by these indirect transmission experiments. This model is capable of accurately describing the pathogen dispersal process by a diffusive transport mechanism which includes pathogen mortality. Lastly, a range of dose-response models were compared and tested how well these fitted to the data from a dose-response experiment. Here it was shown that for interpolation purposes two relatively simple models are best capable of describing the data from the dose-response experiment. For extrapolation purposes, however, it was shown that from the models that were studied a model that abides by the independent action hypothesis is best.

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CHAPTER 1

General Introduction

Infectious disease transmission

Infectious diseases are caused by pathogenic (micro-) organisms such as bacteria, viruses, parasites or fungi and infectious diseases are widespread in both the plant and animal kingdom (including humans) [1-9]. These diseases have a major impact worldwide, not only on the health and welfare of humans and animals, but also on the economy [10].

Direct transmission

Truly direct transmission, i.e. transmission by direct contact, is limited to vertical transmission, sexually transmitted diseases and diseases like Human Papillomavirus, Epstein-Barr virus or Cytomegalovirus. Other routes of transmission that exist are: respiratory routes, often termed airborne transmission (influenza, tuberculosis or measles), faecal-oral routes, where water or foodstuff gets contaminated, or vector-borne routes, where the actual transmission takes place via a vector. All these routes of transmission consist of an environmental stage and are in its strictest sense not direct transmission but indirect transmission. Transmission can occur without the need for physical contact between an infectious sender and a susceptible receiver. The difference between direct transmission and indirect transmission is thus not always very clear for many diseases and most transmission events that are approximated as direct are in fact indirect. In many situations this approximation is valid and we can treat this form of indirect transmission as if it were direct transmission. However there are examples in which we cannot (safely) ignore the environmental stage and actively have to incorporate an environmental stage if we want to use mathematical models to describe the spread of a disease accurately or have accurate parameter estimates [11, 12].

Indirect transmission

Indirect transmission can be thought of as a process consisting of three separate sub-processes:

1. an infectious sender that excretes infectious material;
2. traveling of the infectious material through the environment outside the host, getting from sender to receiver via some route; and
3. a susceptible receiver that takes in (some) of the infectious material and is getting infected by this material (Figure 1).

By dividing this process in three sub-processes these individual parts can be studied separately, thus avoiding having to deal with complicated interaction effects. Breban et al and Rohani et al [13, 14] investigated the possibility of indirect transmission of low pathogen Avian Influenze (LPAI) between wild waterfowl by including the environment outside the host

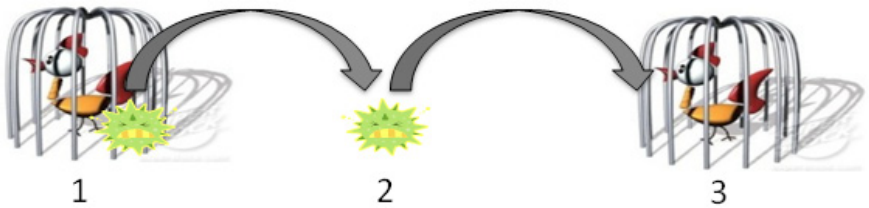

Figure 1. Indirect transmission can be divided in three separate sub-processes. 1: an infectious sender excreting invfectious material. 2: transport of infectious material through the environment. 3: uptake of infectious material by a susceptible receiver.

as a reservoir i.e. sub-process 2 of the above mentioned sub-processes. In their studies they found that environmental transmission can be an explanation for the recurrent outbreaks of LPAI every 2-4 years. Their findings suggest that indirect transmission provides a persistent mechanism within small communities where an epidemic cannot be sustained by direct transmission alone. Another approach followed by Boender *et al* [6] (amongst others) is to capture all (mostly unknown) routes in one distance dependent kernel. This appears to be a very elegant and fruitful approach. With this approach it is still possible to have quantitative estimation for many outbreak parameters without the need to know the actual route that has been followed from one infected animal to the next.
A number of studies have included indirect transmission in the recent past to quantify transmission between spatially separated animals [12, 15, 16] but none of them give any insight in the likely mechanisms that underlie (indirect) transmission and they do not explicitly take temporal patterns or effects into account.

Neighbourhood transmission

Highly transmissible diseases of livestock transmit not only from animal to animal which could be considered direct transmission but also from farm to farm where it is clear that the transmission is indirectly unless transmission is by animals being moved between farms. Numerous routes are identified for the transmission between farms [17-22], and there is some published data on specific routes of between-farm transmission [23-25]. A major complicating factor during major outbreaks is that in all recent epidemics, between-farm infections continued to occur in spite having bans on all contact between farms (both movement bans, bans on visitors and shared equipment). For most of these infections no route could be traced and different studies show that the risk of transmission via these untraced routes declines with increasing distance [6, 17, 18, 26]. These transmission events where therefore often coined as “neighbourhood infections” [5, 18, 27] and were responsible for the majority of the infections (estimations range from 60-80% [28]) during the recent major outbreaks of Classical Swine Fever Virus (CSFV) in 1997/1998. This neighbourhood transmission is indirect transmission, because there is no direct contact between animals on the different farms when transmission occurs.
To combat and control an outbreak of a highly transmissible livestock disease different instruments are available, for example, culling of animals, vaccination of animals and different bio-security measures such as a ban on all transport of animals and animal products during an outbreak, implementation of hygiene protocols for visitors on and off the farms etc. Data from the major outbreaks in the last decade have proven to be highly valuable for assessing the efficacy of preventive culling and vaccination strategies [29-32], as for the assessment of these measures only the total force of transmission has to be known (estimated from the data). These major outbreaks have enormous socio-economic effects because of bans on livestock movements, animal culling, standstill in trade and export bans [33, 34]. For example, the costs of the FMD outbreak in the UK in 2001 were estimated to be about €4.1 billion [34]. This clearly underlines the need for better understanding of indirect transmission in order to put in place evidence based bio-security measures against (indirect) neighbourhood

transmission. For bio-security measures on the other hand more in depth knowledge is needed about the individual contributions of different underlying transmission routes and for many of the present bio-security measures no evidence-based quantitative estimates are available on the individual contribution towards reducing transmission. A notable exception being the results published by Ssematimba et al [22] where from the comparison of the per contact transmission it was clear that movement of contacts with strict hygiene protocols from an infected farm to a non-infected farm (crisis organisation teams) was much less risky than contacts that occurred with less strict hygiene protocols. Further knowledge about the contribution of the different bio-security measures would help to apply these measures in more optimal way during a disease outbreak, thereby sparing animal lives and saving costs.
Indirect transmission is not only a problem with livestock diseases, it is also very important for diseases that spread between humans [9, 35]. As an example, the spread of many antibiotic resistant bacteria is probably, at least partly, due to indirect transmission between patients.

Approach

To gain better insight in the underlying mechanisms of transmission between spatially separated animals, a quantitative approach was used throughout this thesis. Instead of experimentally studying indirect transmission on farm level, which was not feasible, in this thesis it is studied at a smaller scale with the use of novel tailor-made animal experiments using the transmission of bacteria between broiler chickens as a model system for indirect transmission. By combining the data from these experiments with tailor-made mathematical models it is possible to not only get analytical insights from the mathematical models, but also parameterise the models using experimental data. Furthermore it enables us to test how well the fit of the proposed model is to the experimental data. In the animal experiments used as a model system for indirect transmission, the bacteria used were either Campylobacter jejuni (C. jejuni) or both C. jejuni and Escherichia coli (E. coli). Previous studies show that acidification of feed can have an effect on the spread of C. jejuni or the susceptibility of broiler chickens [36-39], therefore acidification of the drinking water was used as a modulation factor for indirect transmission. This allows us to study the sender and receiver sub-process separately.

Aims and outline of this thesis

The goal of this thesis was to determine the underlying mechanism of transmission between spatially separated hosts. Therefore the effect of acidification of drinking water on both direct and indirect transmission of C. jejuni was studied in chapter 2 to obtain quantitative data on the differences between direct and indirect transmission. The indirect transmission process is then studied in more detail in chapter 3. In this chapter the effect of acidification of the drinking water at the sender and the receiver side in indirect transmission of C. jejuni between spatially separated broilers is studied to get a better understanding of the sub-processes 1 and 3 of indirect transmission (Figure 1). Because the routes of indirect transmission are mostly unknown this sub-process is studied in chapter 4. In this chapter we use data of novel tailor-made indirect transmission experiments to develop a new model framework to help interpreting

a large delay before the first transmission event followed by clustering of transmission events that was found when studying indirect transmission between spatially separated broilers. Chapter 5 describes in detail a two-dimensional diffusion model that explicitly incorporates the distance between hosts and time pathogen spends in the environment. With this model we are able to describe accurately the differences in indirect transmission patterns of C. jejuni and E. coli between spatially separated broilers and a delayed transmission component that exists in the spread of Vancomycin resistant Enterococcus between patients in an intensive care unit. Lastly, chapter 6 describes a range of dose-response models that are fitted to data from dose-response experiments, paying special attention to the fit of these models to low doses.

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CHAPTER 2

Acidification of drinking water inhibits indirect transmission, but not direct transmission of *Campylobacter* between broilers

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Abstract

In this study the effect of acidification of the drinking water of broiler chickens on both direct and indirect transmission of *Campylobacter* was evaluated. In the direct transmission experiment both susceptible and inoculated animals were housed together. In the indirect transmission experiment the susceptible animals were spatially separated from the inoculated animals and no direct animal to animal contact was possible. The transmission parameter β was estimated for the groups supplied with acidified drinking water and for the control groups. The results showed that acidification of the drinking water had no effect on direct transmission ($\beta = 3.7$ day^{-1} for both control and treatment). Indirect transmission however was influenced by acidification of the drinking water. A significant decrease in transmission was observed ($p < 0.05$), with control vs. treatment point estimates being $\beta = 0.075$ day^{-1} vs. $\beta = 0.011$ day^{-1}. Apart from providing quantitative estimations of both direct and indirect transmission of *Campylobacter* in broilers, this study also demonstrates the use of an experimental setup for indirect transmission of *Campylobacter* between broilers to assess the efficacy of candidate measures to reduce transmission.

Introduction

Campylobacter causes a substantial number of cases of human gastroenteritis worldwide [1, 2]. The handling and consumption of contaminated poultry products are major risk factors for *Campylobacteriosis* [3]. Implementation of measures to control *Campylobacter* in the poultry production chain may reduce the exposure of humans to *Campylobacter*. Such measures can be applied either at the slaughterhouse level, i.e. improving the slaughterhouse hygiene, or they can be applied at primary production level, i.e. on farm hygiene and biosecurity measures, to reduce the incidence of *Campylobacter* colonised flocks. A reduction in the number of colonised poultry flocks will decrease the risk for consumers considerably [4]. One way of reducing the number of colonised poultry flocks is by altering the susceptibility of the host; i.e. the chance of successful colonisation after exposure [5]. In broiler chickens, fermented liquid feed (FLF) has been shown to reduce the susceptibility to *Campylobacter* and *Salmonella* [6-8]. In FLF, lactobacilli are present whose main metabolic products are lactic acid and acetic acid [9]. The effects of FLF are attributed to the high level of organic acids and the low pH of this feed. Following this line of reasoning, acidified drinking water may be expected to have a similar effect on the susceptibility of broilers to *Campylobacter* as FLF.

The aims of this study were (1) to investigate the effect of acidification of the drinking water on both the direct and indirect transmission of *Campylobacter* between broilers and (2) to explore the use of an experimental system of indirect transmission of *Campylobacter* between broilers for assessing the effect of candidate measures against transmission in a controlled setting. With indirect transmission we mean transmission that occurs in a situation where there is no possibility for contact between susceptible and infectious animals, i.e. they are spatially separated.

Materials and methods

Experimental design

Direct transmission experiment

The direct transmission experiment consisted of one control group and one treatment group and was carried out in duplicate, resulting in four groups in total. Each group consisted of 23 animals. Throughout the experiment the groups were housed in separate stables. From day 0 (day of hatching) until day 12 all animals in a group were housed together. The two control groups received tap water whereas the treatment groups continuously received acidified drinking water. A commercially available acid (Forticoat®, Selko BV) was diluted until a final pH of 4 (approximately 2 ml acid on 1 litre water). Active ingredients of the commercially available acid are: sorbic acid, formic acid, acetic acid, lactic acid, propionic acid, ammonium formate, L-ascorbic acid, citric acid, mono- and diglycerides of edible fatty acids and 1,2–propanediol. At day 12, ten animals per group were randomly selected from each group, inoculated with *Campylobacter* by gavage (see section on Inoculum) and housed separately. On day 16 the inoculated animals were placed back with the rest of their group. Colonisation was monitored by taking cloacal swabs on a daily basis from day 14 onwards. The swabs were processed within 2 hours for the analysis of the presence of *Campylobacter*. If an animal was found positive on 5 consecutive days, swabs were taken only once a week. The experiment

was ended 20 days post inoculation. At that day all chickens were euthanised and caecal contents were qualitatively analysed for the presence of *Campylobacter*.

Indirect transmission experiment

The indirect transmission experiment consisted of one control group and one treatment group and was carried out in duplicate. Each group consisted of 9 animals. The two control groups received tap water, the treatment groups received acidified drinking water (Forticoat®, Selko BV, pH: 4). From day 0 (day of hatching) until day 4 all animals in a group were housed together. On day 4, animals were housed individually according to the housing plan depicted in Figure 1. This setup was chosen to equalise the infection pressure experienced by each susceptible bird as much as possible. Twelve days after hatching 5 animals from each group were orally inoculated with 1 ml of *Campylobacter* (see section on Inoculum). To monitor colonisation, from day 12 onwards, swabs were taken on a daily basis from all animals, both inoculated and susceptible. If an animal was found positive for *Campylobacter* on 5 consecutive days, swabs from that animal were taken on a weekly basis. The experiment was ended 21 days post inoculation. All animals were euthanised and the caeca were removed and qualitatively analysed for the presence of *Campylobacter*.

Housing

Animals were housed in wire cages placed directly on the floor. Wood shavings were provided as bedding material; feed was supplied ad lib.; drinking water was supplied via an open water drinking system. No flow of water was possible between infectious and susceptible animals. Drinking water was refreshed on a regular basis. Before the start of the experiment all stables used in the experiment were cleaned and disinfected and samples were taken from different areas inside the stable, to check for the absence of *Campylobacter*.

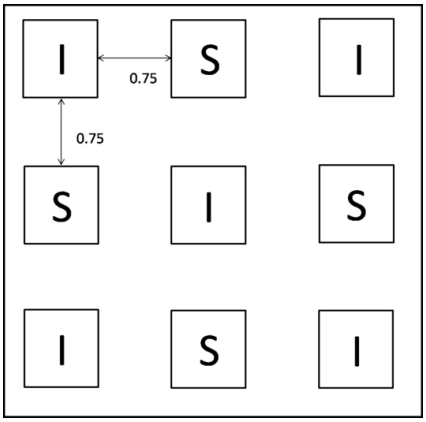

Figure 1. Schematic overview of the housing of the animals during the indirect transmission experiment. S: susceptible animal; I: infectious animal. Distances are given in metres.

Animals

Eggs from commercial broilers (type Ross 308) were incubated in an in-house facility. Day of hatching is day 0 in the experiment. On day 1 and day 8 cloacal swabs were taken from all animals to check for the absence of *Campylobacter*. These samples were incubated in mCCD (modified cefoperazone charcoal deoxycholate) broth (Nutrient Broth no. 2, Oxoid CM0067 with *Campylobacter* selective supplement (Oxoid SR0204E) and *Campylobacter* growth supplement (Oxoid SR0232E)) for 24 hours and plated on mCCDA (modified cefoperazone charcoal deoxycholate agar) and incubated again to check suspected *Campylobacter* colonies after 24 and 48h (see

section on Sampling for complete procedure). All animals were uniquely tagged so they could be tracked throughout the experiment.
All animal experiments were in compliance with national and institutional regulations and as such approved by the institute's ethical committee.

Inoculum

The *Campylobacter* strain used in this experiment was *Campylobacter jejuni* strain C356, originally isolated from broilers [10]. The strains were freshly cultured in hearth infusion broth (microaerobically, 37°C, overnight) and diluted in buffered peptone water to obtain the intended inoculation dose (10^5 CFU/ml).

Sampling

Samples were collected using sterile swabs. Swabs were directly plated on mCCDA, these plates where incubated microaerobically at 41.5°C for 48h and examined for the presence of *Campylobacter*. After plating the swabs were placed in an enrichment medium (CCD broth) and incubated microaerobically at 41.5°C for 24h. After incubation 10 µl was plated on mCCDA and incubated microaerobically at 41.5°C for 48h and examined for the presence of *Campylobacter*. Sensitivity and specificity for testing cloaca swabs were estimated as both being very close to 1 (personal communication R. van der Hulst, CVI, Lelystad).

Quantification of transmission

A stochastic susceptible-infectious (SI) type model was used to describe the transmission between inoculated animals (seeders) and susceptible animals (contact animals). In the SI-model individuals in a population of size N are either susceptible (S) or infected (I). Susceptible individuals get infected with rate βSI/N, where β is the transmission parameter. Substituting S by N-I and given a sufficiently small time interval βt, it is possible to formulate separate differential equations for the probability of finding the population in every possible state [11, 12].
These master equations (or state probabilities) can be written in matrix form: $dP/dt = Q \cdot P$ with generator matrix:

$$Q = \begin{pmatrix} -\beta(N-1)/N & 0 & \cdots & 0 & 0 \\ \beta(N-1)/N & -2\beta(N-2)/N & \cdots & 0 & 0 \\ 0 & 2\beta(N-2)/\mathrm{N} & \cdots & 0 & 0 \\ \vdots & \vdots & \cdots & \vdots & \vdots \\ 0 & 0 & \cdots & -\beta(N-1)/N & 0 \\ 0 & 0 & \cdots & \beta(N-1)/N & 0 \end{pmatrix}$$

and their solution is: $\vec{p}(t) = e^{Qt}\vec{p}_0$. A further explanation and an implementation of this method are given in the appendix.
Using this solution, the probability of the state observed at each sampling moment, conditional on the state observed at the previous sampling moment, can be calculated in an exact manner provided that the sensitivity and specificity of the cloaca swabbing are 1. Thus β can

Table 1. Results for the direct transmission experiment. Total number of observed colonised broilers, corresponding day number of colonised broilers per stable and per treatment estimate of transmission parameter β are shown.

Stable	Type of drinking water	Observed colonised broilers (Total animals)	Day numbers of observed colonised broilers (p.i.)	β (95% C.I.)
1	Normal tap water	13 (13)	1	n.a.[a] (2.5 - ∞)
2	Normal tap water	13 (13)	1	
3	Acidified tap water	13 (13)	1,2	3.7 (2.0 - 6.8)
4	Acidified tap water	13 (13)	1	

[a] **All** animals were found positive for Campylobacter on the first day of sampling, therefore, the point estimate for the transmission parameter is unidentifiable from the available data. p.i.: post inoculation, C.I.: confidence interval.

be estimated using maximum likelihood and exact confidence bounds can be obtained by summing the probabilities of all scenarios that are as extreme as or more extreme than the observed scenario. All calculations were implemented and performed in Mathematica 7.0 [13].

Results

The results of the direct transmission experiment are given in Table 1. For both control and treatment, after the inoculated (and infectious) animals were placed back with the susceptible animals, all susceptible animals became colonised with *Campylobacter*. The colonisation of the contact animals occurred rapidly: within 1 day for all but one (an animal in one of the treatment groups which became colonised on day 2). The estimation of the transmission parameter β with the maximum likelihood procedure yielded a value of 3.7 (95% C.I.: 2.0 – 6.8) per day. No significant differences in the transmission parameter were found between control and treatment groups (Wald-test, p = 0.9) and the data of the two groups were pooled in subsequent analyses.

For the indirect transmission experiment the number of transmission events (colonisation) per stable is given in Table 2. These results show that indeed indirect transmission of *Campylobacter* between spatially separated broilers occurred. Furthermore fewer colonised animals in the treatment stables compared to the control stables were found and, when tested with a one-sided Fisher exact test, this difference is found to be significant (Fisher exact, p=0.035). A one sided test was used here because we did expect less animals to be colonised in the treatment stables. One animal died in stable 2; this animal was excluded from the analysis.

The estimates obtained for the transmission parameter β are shown in the last column of Table 2. For the control groups we found an estimate for β of 0.099 (95% C.I.: 0.035-0.21)

Table 2. Results for the indirect transmission experiment. Total number of observed colonised broilers, corresponding day number of colonised broilers per stable and per treatment estimate of transmission parameter β are shown.

Stable	Type of drinking water	Observed colonised broilers (Total animals)	Day numbers of observed colonised broilers (p.i.)	β (95% C.I.)
1	Normal tap water	3 (4)	12,12,14	0.075 (0.027 - 0.16)
2	Normal tap water	2 (3)	15,18	
3	Acidified tap water	1 (4)	20	0.011 (0.0006 - 0.047)
4	Acidified tap water	0 (4)	–	

p.i.: post inoculation, C.I.: confidence interval.

per day. The estimate found for the treatment groups was 0.011 (95% C.I.: 0.0006-0.049) per day. Based on a Wald-test the difference between the control and treatment groups was significant ($p<0.05$), indicating that acidification of the drinking water reduced the transmission parameter.

Discussion

This study was carried out to determine whether acidification of the drinking water has an influence on the transmission of *Campylobacter*. Both direct and indirect transmission (transmission between spatially separated broilers) was investigated. As we used a novel experimental setup with spatially separated broilers to study indirect transmission, this study also served to explore its use as a system to test possible measures to reduce indirect transmission.

Our results showed that acidification of the drinking water had no effect on the direct transmission of *Campylobacter* between broilers; however, there was a significant reduction in transmission between spatially separated broilers (i.e. indirect transmission) when the drinking water was acidified.

Three hypotheses may explain the effect of acidification of the drinking water on indirect transmission. First, the host animal might be less susceptible for *Campylobacter* colonisation due to acidification of the drinking water. The basis of this is that gizzard and stomach of the chickens become more acidic when the animals receive acidified drinking water. This might reduce the number of bacteria that reach the lower parts of the gastrointestinal tract. Bjerrum *et al* demonstrated a similar effect in broilers fed with whole wheat [14], which has an acidifying effect in the gizzard. They showed that broilers fed with whole wheat had significantly reduced numbers of *Salmonella typhimurium* in the gizzard and ileum. However no difference with respect to the number of *Salmonella* was found in the caeca and rectum. As a second hypothesis there is the possibility that due to the acidification of the drinking water, the actual number of bacteria per gram faeces shedded by the inoculated animals is less compared to a control situation, eventually leading to a decreased probability of colonisation of the susceptible animals. A third hypothesis involves the environment the bacteria pass through on their way from the shedding animal to the receiving susceptible animal. Once shedded via the faeces, the bacteria enter a more hostile environment, due to the acidification, and the dying off in the environment increases, thereby decreasing the probability of colonisation. With the current experiment we cannot distinguish between these three hypotheses and further research is needed to identify the correct mechanism(s). As the three hypotheses are not mutually exclusive also a combination of two or three is possible as an explanation for the effect observed.

Also the exact route of indirect transmission remains unknown. For example, dust, litter or animal care-takers are just some possible routes of transmission. More research about the exact routes of transmission is needed to determine and classify routes of indirect transmission. Indeed for some infectious diseases this has been attempted [15-17], however for many diseases the actual routes and the contribution of the different routes remain unclear. As the indirect transmission experiment carried out in this study mimics a between-flock

transmission situation, the findings may indicate that acidification of the drinking water might have a reducing effect on between-flock transmission. In a modelling analysis of interventions, Katsma *et al* showed that the most effective method to reduce the *Campylobacter* prevalence is to reduce the between-flock transmission [18]. This underlines the potential effect of acidification of the drinking water as a possible control measure. It should be noted however that our direct transmission experiment showed that the direct transmission of *Campylobacter* is not affected by acidification of the drinking water. Therefore once *Campylobacter* is introduced into a flock it will still spread fast within this flock ($\beta = 3.7$ day^{-1}), although some care must be taken when extrapolating from an experimental setup as in this study to a full commercial flock. It should be noted that the sample size in this study is relatively small, resulting in the parameter estimation being sensitive to small differences in number of infected animals. To get more robust parameter estimates more replications of this study should be performed.

The main conclusions of these experiments are that direct transmission (within-flock transmission) of *Campylobacter* between broilers is not altered by acidification of the drinking water; however, acidification of the drinking water has a decreasing effect on the indirect transmission of *Campylobacter* between broilers. Whether this effect is large enough to contribute meaningfully to the transmission of *Campylobacter* between flocks needs to be studied under field conditions. The results of these experiments also show that our experimental setup for indirect transmission of *Campylobacter* between broilers is a promising approach for evaluating candidate measures for the reduction of transmission.

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Appendix

Estimation of the transmission parameter in the stochastic SI-model using master equations.

In the SI-model individuals are either susceptible (S) or infectious (I). The rate at which an infection event occurs is $\beta SI/N$, where β is the transmission parameter and N is the total number of individuals. Thus when the system is in the state [S,I] it moves to the state [S-1,I+1] with rate $\beta SI/N$. We assume that we have observed the state of the population at discrete time points, t, $t+\Delta t$, etc. Let $S(t)$ denote the number of susceptible animals at time t, $I(t)$ the number of infectious animals. Assuming for simplicity that N is constant in time, since $S(t) = N - I(t)$, the system is fully described by $I(t)$. Assuming that the state of the system is observed at time t, we denote $n_I \equiv I(t)$ and analyse the changes in n_I with time as follows:

For the population to be in state n_I at time $t+\Delta t$, either it is in state n_I at time t and no infection event occurred during t or it was in state n_I-1 at time t and an infection event occurred during t. The probability that the population will go from n_I to n_I+1 is approximately $\beta(N-n_I)n_I\beta t/N$ and the probability that it stays in the same state is $1-\beta(N-n_I)n_I\beta t/N$; that is,

$$p_{n_I}(t+\Delta t) = p_{n_I-1}(t)\beta\frac{(N-n_I-1)(n_I-1)}{N}\Delta t + p_{n_I}(t)(1-\beta\frac{(N-n_I)n_I}{N}\Delta t$$

Denoting $\lambda_n \equiv \beta(N-n)n/N$ (the force of infection), subtracting p_n from both sides, dividing by t and letting $t \to 0$, results in a system of differential equations:

$$\frac{dp_n(t)}{dt} = \lambda_{n-1}p_{n-1}(t) - \lambda_n p_n(t), \text{ for } n = 1,2,\ldots,N$$

This can also be written as $d\vec{p}/dt = Q\vec{p}$, with Q being the generator matrix (see Allen, 2010 for the denition and construction of the generator matrix Q, which is beyond the scope of this appendix). For the SI-model the generator matrix is

$$Q = \begin{pmatrix} -\beta(N-1)/N & 0 & \cdots & 0 & 0 \\ \beta(N-1)/N & -2\beta(N-2)/N & \cdots & 0 & 0 \\ 0 & 2\beta(N-2)/\mathrm{N} & \cdots & 0 & 0 \\ \vdots & \vdots & \cdots & \vdots & \vdots \\ 0 & 0 & \cdots & -\beta(N-1)/N & 0 \\ 0 & 0 & \cdots & \beta(N-1)/N & 0 \end{pmatrix}$$

This is a system of differential equations for which the matrix exponential yields the general solution: $M = e^{Qt}$. Obtaining the matrix exponential can be quite complicated for larger matrices, however most mathematical software packages have routines to calculate the matrix exponential. If we apply the general solution to a vector describing our initial conditions we get a particular solution: $\vec{p}(t) = e^{Qt}\vec{p}_0$.

With this solution we can formulate the parameter likelihood given the observations. Therefore we let each observation contribute to the overall likelihood. The overall likelihood for an experiment is thus of the form:

$$L = \prod_{i=0}^{n} p_{m_i}(t_i)$$

where n is the total number of observations, p_{mi} indicates that we take element number m_i from p, where m is a list with the number of infected animals at each observation, and finally t is a

list of time periods between the observations. Note that for each state change a different $\vec{p}(t)$ needs to be calculated due to a different $\vec{p}_0$.

Given the observations we can calculate all the probabilities $p_{t,t+\Delta t}$ by substituting the appropriate values for the numbers *N*, *I(t)*, and *S(t)*, and estimate by maximising the likelihood with respect to β. As an example we will use the above recipe to estimate for stable 1 of the normal tap water treatment of the indirect transmission experiment. Here $N = 9$, $S_0 = 4$, $I_0 = 5$, and 2 infections occurred at day 12 and 1 infection at day 14.

First we can calculate generator matrix *Q*:

$$Q = \begin{pmatrix} -\frac{8\beta}{9} & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 \\ \frac{8\beta}{9} & -\frac{14\beta}{9} & 0 & 0 & 0 & 0 & 0 & 0 & 0 \\ 0 & \frac{14\beta}{9} & -2\beta & 0 & 0 & 0 & 0 & 0 & 0 \\ 0 & 0 & 2\beta & -\frac{20\beta}{9} & 0 & 0 & 0 & 0 & 0 \\ 0 & 0 & 0 & \frac{20\beta}{9} & -\frac{20\beta}{9} & 0 & 0 & 0 & 0 \\ 0 & 0 & 0 & 0 & \frac{20\beta}{9} & -2\beta & 0 & 0 & 0 \\ 0 & 0 & 0 & 0 & 0 & 2\beta & -\frac{14\beta}{9} & 0 & 0 \\ 0 & 0 & 0 & 0 & 0 & 0 & \frac{14\beta}{9} & -\frac{8\beta}{9} & 0 \\ 0 & 0 & 0 & 0 & 0 & 0 & 0 & \frac{8\beta}{9} & 0 \end{pmatrix}$$

From the data we know the system has been in three separate states: a state with 5 infected animals, a state with 7 infected animals and a state with 8 infected animals.

Thus we have to calculate $\vec{p}', \vec{p}''$ and $\vec{p}'''$, all with different $\vec{p}_0$.

We start at the state $I_0 = 5$, i.e. $\vec{p}_0 = \begin{pmatrix} 0 \\ 0 \\ 0 \\ 0 \\ 1 \\ 0 \\ 0 \\ 0 \\ 0 \end{pmatrix}$,

applying the matrix exponential at $\vec{p}_0$ gives:

$$\vec{p}'(t) = \begin{pmatrix} 0 \\ 0 \\ 0 \\ 0 \\ e^{-\frac{20t\beta}{9}} \\ 10e^{-\frac{20t\beta}{9}}\left(-1+e^{\frac{2t\beta}{9}}\right) \\ 15e^{-\frac{20t\beta}{9}}\left(-1+e^{\frac{2t\beta}{9}}\right)^2\left(2+e^{\frac{2t\beta}{9}}\right) \\ 7e^{-\frac{20t\beta}{9}}\left(-1+e^{\frac{2t\beta}{9}}\right)^3\left(5+6e^{\frac{2t\beta}{9}}+3e^{\frac{4t\beta}{9}}+e^{\frac{2t\beta}{3}}\right) \\ 1+14e^{-\frac{20t\beta}{9}}-28e^{-2t\beta}+20e^{-\frac{14t\beta}{9}}-7e^{-\frac{8t\beta}{9}} \end{pmatrix}$$

For the state with $I=7, \vec{p}_0 = \begin{pmatrix} 0 \\ 0 \\ 0 \\ 0 \\ 0 \\ 0 \\ 1 \\ 0 \\ 0 \end{pmatrix}$, and $\vec{p}''(t) = \begin{pmatrix} 0 \\ 0 \\ 0 \\ 0 \\ 0 \\ 0 \\ e^{-\frac{14t\beta}{9}} \\ \frac{7}{3}e^{-\frac{14t\beta}{9}}\left(-1+e^{\frac{2t\beta}{3}}\right) \\ 1+\frac{4}{3}e^{-\frac{14t\beta}{9}}-\frac{7}{3}e^{-\frac{8t\beta}{9}} \end{pmatrix}$

And finally for the state $I=8, \vec{p}_0 = \begin{pmatrix} 0 \\ 0 \\ 0 \\ 0 \\ 0 \\ 0 \\ 0 \\ 1 \\ 0 \end{pmatrix}$, and $\vec{p}'''(t) = \begin{pmatrix} 0 \\ 0 \\ 0 \\ 0 \\ 0 \\ 0 \\ 0 \\ e^{-\frac{8t\beta}{9}} \\ e^{-\frac{8t\beta}{9}}\left(-1+e^{\frac{8t\beta}{9}}\right) \end{pmatrix}$

To estimate β for this stable we have to multiply the probabilities of all observations and maximize this expression with respect to β. Taking the likelihood function we defined earlier we can now easily fill this out:
We start with 11 days post inoculation (p.i.) in a state with 5 infected animals, thus we evaluate element 5 of $\vec{p}'$ at $t = 11 : e^{-\frac{20 \cdot 11 \cdot \beta}{9}}$

Then at day 12 p.i. (1 day after the last observation) the system changes to a state with 7 infected animals, i.e. we evaluate element 7 of $\vec{p}'$ at $t = 1 : \; 15e^{-\frac{20\beta}{9}} \left(-1 + e^{\frac{2\beta}{9}} \right)^2 \left(2 + e^{\frac{2\beta}{9}} \right)$

From here on our $\vec{p}_0$ has changed and we need to use $\vec{p}''$.

At day 13 p.i. the system stays in the state with 7 infected animals: evaluate element 7 of $\vec{p}''$ at $t = 1 : \; e^{-\frac{14\beta}{9}}$

At day 14 p.i. the system changes to a state with 8 infected animals: evaluate element 8 of $\vec{p}''$ at $t = 1 : \; \frac{7}{3} e^{-\frac{14\beta}{9}} \left(-1 + e^{\frac{2\beta}{3}} \right)$

Finally the system remains in a state with 8 infected animals until day 21 p.i. (the end of the experiment), i.e. evaluate element 8 of $\vec{p}'''$ at $t = 7 : \; e^{-\frac{8 \cdot 7 \cdot \beta}{9}}$
Multiplying all terms results in:

$$L = e^{-\frac{220 \cdot \beta}{9}} \cdot 15e^{-\frac{20 \cdot \beta}{9}} \left(-1 + e^{\frac{2 \cdot \beta}{9}} \right)^2 \left(2 + e^{\frac{2 \cdot \beta}{9}} \right) \cdot e^{-\frac{14 \cdot \beta}{9}} \cdot \frac{7}{3} e^{-\frac{14 \cdot \beta}{9}} \left(-1 + e^{\frac{2 \cdot \beta}{3}} \right) \cdot e^{-\frac{56 \cdot \beta}{9}}$$

Maximising this function for β yields β = 0.085.

CHAPTER 3

Interaction effects between sender and receiver processes in indirect transmission of *Campylobacter jejuni* between broilers

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Abstract

Background: Infectious diseases in plants, animals and humans are often transmitted indirectly between hosts (or between groups of hosts), i.e. via some route through the environment instead of via direct contacts between these hosts. Here we study indirect transmission experimentally, using transmission of *Campylobacter jejuni* (*C. jejuni*) between spatially separated broilers as a model system. We distinguish three stages in the process of indirect transmission; (1) an infectious "sender" excretes the agent, after which (2) the agent is transported via some route to a susceptible "receiver", and subsequently (3) the receiver becomes colonised by the agent. The role of the sender and receiver side (stage 1 and stage 3) was studied here by using acidification of the drinking water as a modulation mechanism.

Results: In the experiment one control group and three treatment groups were monitored for the presence of *C. jejuni* by taking daily cloacal swabs. The three treatments consisted of acidification of the drinking water of the inoculated animals (the senders), acidification of the drinking water of the susceptible animals (the receivers) or acidification of the drinking water of both inoculated and susceptible animals. In the control group 12 animals got colonised out of a possible 40, in each treatment groups 3 animals out of a possible 40 were found colonised with *C. jejuni*.

Conclusions: The results of the experiments show a significant decrease in transmission rate (β) between the control groups and treatment groups ($p < 0.01$ for all groups) but not between different treatments; there is a significant negative interaction effect when both the sender and the receiver group receive acidified drinking water ($p = 0.01$). This negative interaction effect could be due to selection of bacteria already at the sender side thereby diminishing the effect of acidification at the receiver side.

Introduction

Many infectious diseases, both plant related and animal related (including human diseases) spread via indirect transmission instead of direct transmission. For many plant diseases this process is well understood in terms of fungal spores travelling from one host to the next [1,2]. However for animal diseases indirect transmission is not well understood. For a number of these diseases we have some information on the routes of indirect transmission. For example, in the context of between-farm transmission of infection, indirect pathways such as sharing of equipment and between-farm movement of vehicles and humans are reported as possible routes of transmission [3-7]. Also for a number of human infections (for example hospital infections such as methicillin-resistant *Staphylococcus aureus*) indirect transmission has been implicated. Typically there is a lack of insight into the detailed mechanisms underlying indirect transmission. More insight would help to develop better prevention measures against this form of transmission.

In a simple tentative representation the process of indirect transmission can be thought of as consisting of three stages. As a first stage there is an infectious host (the sender) that excretes an agent in the environment. During stage two, the agent has to travel through the environment (via some route or multiple routes) to the susceptible host (the receiver) that can become infected or colonised by the agent in stage three. Using this representation in stages as a reference frame helps us to study how these sub-processes connect and, possibly, interact with each other, thus improving our understanding of the mechanisms of indirect transmission.

In this study we consider only stage 1 and 3 of our representation of indirect transmission. For this study an indirect transmission experiment was carried out. As a model system for indirect transmission we used the spread of *Campylobacter jejuni* (*C. jejuni*) between spatially separated broiler chickens. For colonisation with *C. jejuni* the faecal-oral route is the most likely route of transmission. The faecal-oral route consists mainly of indirect transmission, making this system a suitable model system for studying indirect transmission. Furthermore, we know from previous studies that the rate of indirect transmission can be decreased by acidification of the drinking water [8-10]. Here we used this intervention to obtain more insight into the different stages of indirect transmission and their possible interaction. In the experiment we used a novel setup consisting of three treatment groups, one group in which the (infectious) sender animals received acidified drinking water, one group in which the (susceptible) receiving animals received acidified drinking water and one group in which both sender and receiving animals were given acidified drinking water. From the experimental observations the per day chance of colonisation, the effect of acidification of the drinking water, both at the sender and at the receiver stage, and possible interaction effects between acidification of the sender stage and the receiver stage were estimated.

Methods

Experimental design

Each experiment consisted of one control group and three treatment groups. The experiment was replicated four times. In each group, five chicks were orally inoculated with *C. jejuni* by

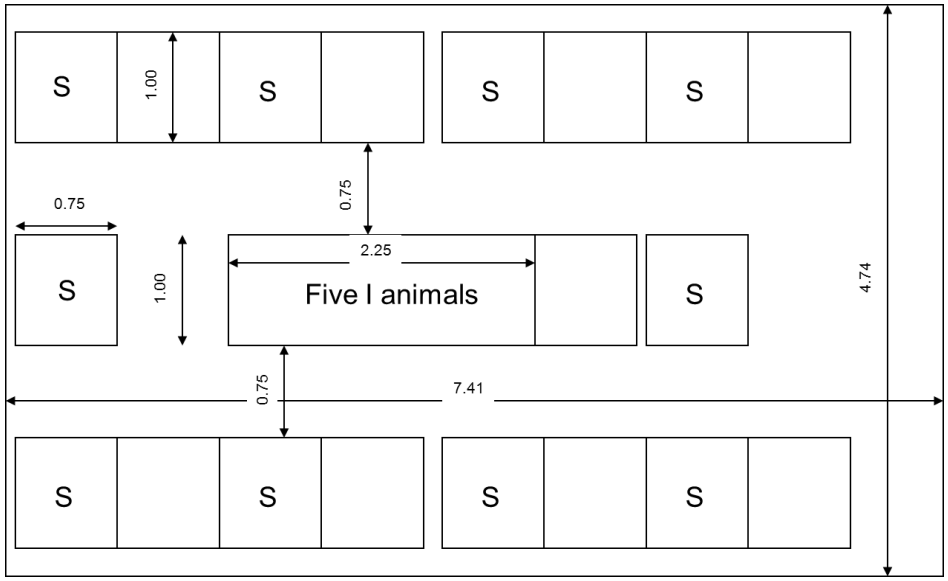

Figure 1. Schematic overview of the housing of the experimental groups of five infectious sender animals (denoted with I) in the centre cage and ten susceptible receiver animals (denoted with S) in the cages surrounding this centre cage. Alongside the arrows distances are given in meters.

gavage. The five inoculated chicks (sender animals) were housed together in one cage in the centre of an experimental room (a climate controlled room in an experimental facility). Ten chicks (receiver animals) were housed individually in cages surrounding this centre cage placed at a minimum distance of 75 cm (see Figure 1) and exposed indirectly to the inoculated sender animals.

The three different treatments were as follows:

1. Acidification of the drinking water of the susceptible animals (indicated as S+);
2. Acidification of the drinking water of the inoculated animals (indicated as I+);
3. Acidification of the drinking water of both inoculated animals and susceptible animals (indicated as S+I+).

To measure indirect transmission, all source and recipient animals were sampled daily by means of a cloacae swab (see section on Sampling). These swabs were tested within two hours after sampling in the laboratory for the presence of *C. jejuni*. If a tested recipient animal was found *C. jejuni* positive, the animal was considered colonised and was immediately removed from the experiment to avoid having to deal in the analysis with multiple cages contributing to the infection pressure. The removed animals were euthanised and cecum was removed for further investigation for the presence of *C. jejuni*.

The experiment ended 35 days post inoculation. All remaining sender and receiver animals (that had not been found positive until that moment) were euthanised and cecum was removed and further investigated for the presence of *C. jejuni*. All animal experiments were in

compliance with national and institutional regulations and as such approved by the institute's ethical committee.

Housing

One-day old broilers (type Ross 308) were obtained from a commercial hatchery. At day 7 and day 12 after arrival, cloacal swabs taken from each chick confirmed the absence of *C. jejuni*. For each of the four experiments from the day of arrival (day 0) until 12 days post-arrival, 60 chicks were housed together in one experimental room, divided in two groups of 30 animals. One group received tap water, the other acidified drinking water. On day 12, the control groups and the treatment groups were formed from the two groups, i.e. for the S+ group 10 animals were randomly taken from the acidified drinking water group and 5 animals from the tap water group; for the I+ group 10 animals were randomly picked from the tap water group and 5 from the acidified drinking water group; for the S+I+ group 15 animals were taken from the acidified drinking water group; and finally for the control group 15 animals were taken from the tap water group. Each treatment group and the control group was placed in its own experimental room, five chicks (sender animals) housed together in one centre cage and ten chicks (receiver animals) individually housed in ten cages surrounding the centre cage as shown in Figure 1. The cages were placed directly on the floor.

All chicks were housed on wood shavings and the drinking water was supplied through a nipple drinking system. In each set-up, the drinking nipples in the cages on the long sides of the area were supplied from one common water container each, while the centre cage and the two cages along the short side each had a separate drinking water supply. This precluded transmission via a shared drinking water system.

Inoculation

For inoculation, the *C. jejuni* strain 356 [11] was used. The strain was freshly cultured in hearth infusion broth (microaerobically, 37°C, overnight) and diluted in buffered peptone water to obtain the intended inoculation dose (± $1 \cdot 10^6$ CFU/ml). The precise concentration (CFU/ml) of *C. jejuni* in the administered inoculum was determined by plating on modified cephoperazone charcoal deoxycholate agar (mCCDA) (Oxoid CM 793) with selective supplement (Oxoid CM 155) before and after the inoculation of the animals. Sender animals were inoculated 14 days after arrival with 1 ml inoculum. All animals were tested positive for *C. jejuni* within 2 days after inoculation.

Treatment

For the acidification of the drinking water a commercial acid (Forticoat®, Selko BV) was diluted until a final pH of 4 (approximately 2 ml acid on 1 litre water).

Active ingredients of the commercial acid are: sorbic acid, formic acid, acetic acid, lactic acid, propionic acid, ammonium formate, L-ascorbic acid, citric acid, mono- and diglycerides of edible fatty acids and 1,2–propanediol.

Sampling and testing

To measure indirect transmission, all animals were tested by means of a cloacae swab. After an inoculated chick (sender animal) was found positive for *C. jejuni* on three consecutive days, swabs for those chicks were taken weekly instead of daily. For the susceptible chicks (receiver animals) swabs were taken once a day throughout the experiment. On days when both inoculated and susceptible animals were to be sampled in each group, the susceptible animals were sampled first. Animals were sampled every day in a fixed order. If a receiver animal tested positive for *C. jejuni*, the animal was immediately removed from the experiment and sacrificed for further investigation of the cecum.

Samples were collected using sterile swabs (sterile plain dry swabs, Copan Diagnostics Inc., USA). Swabs were directly plated on mCCDA, incubated microaerobically at 41.5°C for 48 hours and examined for the presence of *C. jejuni*. The swab was then placed in Preston enrichment medium (Nutrient Broth no. 2, Oxoid CM0067 with *Campylobacter* selective supplement (Oxiod SR0204E) and *Campylobacter* growth supplement (Oxoid SR0232E)) and incubated microaerobically at 41.5°C for 24 hours. After incubation, it was plated on mCCDA and incubated microaerobically at 41.5°C and examined for the presence of *C. jejuni* after 24 and 48 hours.

Hygienic measures

Before the start of the experiment, all experimental rooms were cleaned and disinfected with formaldehyde. Subsequently, samples were taken from 12 different areas inside the room to check for the absence of *C. jejuni*.

To prevent animal caretakers from acting as a vector of transmission, during the entire experiment strict hygienic measures were used. Clean overalls were used at every entry into the experimental rooms. A pair of boots was dedicated to each room, cleaned on entering and exiting it by means of wading through a chlorinated bath (Suma Tab D4, JohnsonDiversity). Sterile gloves were changed between handling individual animals.

Quantification of transmission

Differences in total number of infected animals were tested using a Fisher Exact test. To quantify the transmission between sender and receiver animals a stochastic susceptible-infectious (SI) type model [12] was used. This model can be written in terms of state changes; i.e. if a susceptible receiver animal in the experiment becomes colonised, and is subsequently removed when found positive, we can denote this as $S \rightarrow S\text{-}1$. The rate of this state change is βSI, with a different β for each treatment. From the experimental observations the parameter β was estimated for the different treatments as in [13]. In addition, an analysis of the interaction, if any, between acidification of the sender side or the receiver side was carried out. This latter analysis uses a multiplicative model (additive on log-scale) for the effect of treatments and their possible interaction. Estimation of β was carried out by means of a Generalised Linear Model (GLM) [14]. To this end the data from all repetitions were pooled and represented in the form of $(S(t), C(t), \Delta t)$, where $S(t)$ is the number of susceptible receiver animals at the beginning of a time period with length Δt, $C(t)$ is the number of new colonisations that occurred

Table 1. Number of positive broilers per experiment repetition and total number of exposed animals per treatment group.

Treatment	Repetition				Total positive	Total exposed
	1	2	3	4		
Control	9	2	1	0	12	39†
S+	1	1	0	1	3	40
I+	1	0	0	2	3	40
S + I+	1	2	0	0	3	40

†:One animal died during the experiment.

S+ indicates acidification of the drinking water of the susceptible side. I+ indicates acidification of the drinking water of the infectious side and S+I+ indicates acidification of the drinking water of both susceptible and infectious animals.

in the time period (t, t + Δt). In our model the number of new cases is binomially distributed: $C(t, t+\Delta t) \sim Bin(S(t), p_{inf}(t, t+\Delta t))$,

with parameter $p_{inf}(t, t+\Delta t) = 1 - \exp(-\beta I_{treatment} \Delta t)$ and binomial totals *S(t)*.

This can be rewritten as a GLM with a complementary log-log link function and $\log(I_{treatment}\Delta t)$ as the offset variable [14-16]. We note that because the number of infectious animals is constant over time and new colonisations are removed upon detection, in this setup the estimate for the transmission parameter β is equivalent to the force of infection ($\beta \cdot I_0$).

Results

Table 1 shows the number of colonised animals per treatment group per repetition of the experiment and the total number of colonised animals per treatment. The control group received tap water, while the treatment groups received acidified drinking water at either the sender side, the receiver side or both. In total we observed twelve transmission events in the control group and three transmission events in each treatment group. One susceptible animal died in the control group. Analysis of these overall data shows a significant reduction in transmission between inoculated sender animals and exposed receiver animals for the treatment groups compared with the control group ($p<0.01$ for all groups, Fisher Exact Test). No significant differences in transmission were found between the three treatment groups. We found no correlation between the spatial order of colonisation of recipient animals and the order of sampling of the animals. Figure 2 shows the distribution of transmission events in time. For all groups the transmission parameter β was calculated by GLM from these data. The results are shown in Table 2. For the control group the probability per day of infection (β) was found to be 0.00175 day^{-1} and for each treatment groups 0.00044 day^{-1}. No significant difference was found between the three treatments. This indicates that when one side is acidified there is no additional effect of acidification at the other side. This finding is confirmed by analysing the data as a multiplicative model, which yields a significant negative interaction effect. The results of this test are given in Table 3. A negative interaction effect means that acidifying the drinking water of both sides has less effect than the

Table 2. Estimation of the per day chance of infection for different treatment groups

Treatment	Estimate of β (CI)
Control	0.00175 (0.00129 - 0.00239)
S+	0.00044 (0.00023 - 0.00085)
I+	0.00044 (0.00023 - 0.00087)
S+I+	0.00044 (0.00022 - 0.00085)

S+ indicates acidification of the drinking water of the susceptible side. I+ indicates acidification of the drinking water of the infectious side and S+I+ indicates acidification of the drinking water of both susceptible and infectious animals. CI = 95% confidence interval.

Table 3. Interaction effects between receiver and sender treatment.

Group	Estimate	Std. Error	p
Control	−6.346	0.155	<.001
S+	−1.368	0.333	<.001
I+	−1.388	0.333	<.001
S+I+	1.362	0.534	0.011

S+ indicates acidification of the drinking water of the susceptible side. I+ indicates acidification of the drinking water of the infectious side and S+I+ indicates acidification of the drinking water of both susceptible and infectious animals. Estimates given are for the natural logarithm of multiplicative effects on the transmission parameter.

multiplication (addition on a log-scale) of the two one-side acidification effects. The small difference in the Akaikes Information Criterion (AIC) for the univariate model (AIC = 186.31) and the model with interactions (AIC= 186.59) suggests that, although the interaction effect is significant, it does not improve the model fit and thus interaction is not necessary to explain the data [17].

Discussion

The role of the sender and receiver was studied here by using indirect transmission of *C. jejuni* between spatially separated broilers as a model system with acidification of the drinking water as a modulation factor.

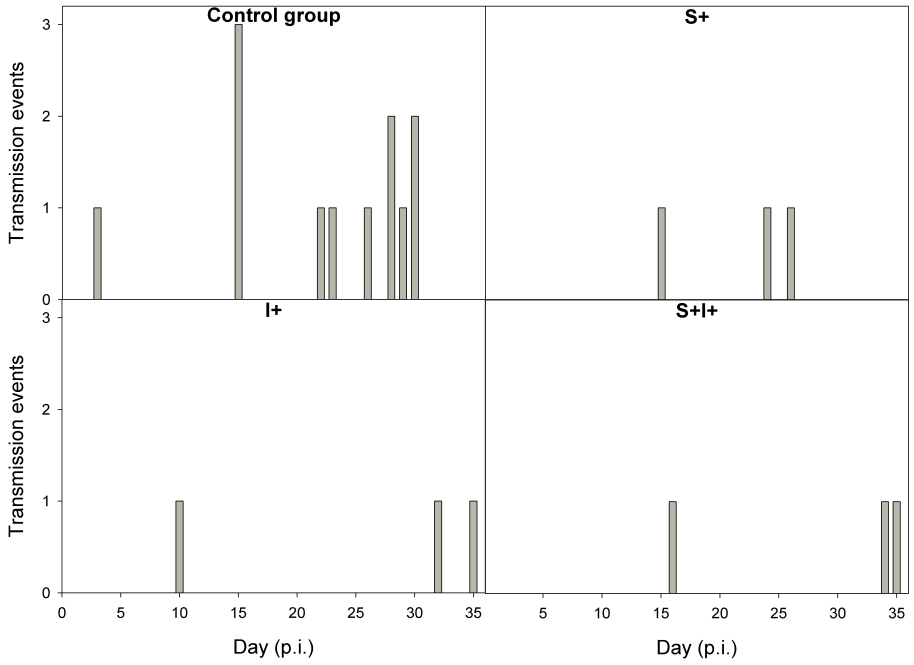

Figure 2. Experimental results showing the number of new infections per treatment group per day after inoculation.

S+ indicates acidification of the drinking water of the susceptible side. I+ indicates acidification of the drinking water of the infectious side and S+I+ indicates acidification of the drinking water of both susceptible and infectious animals, p.i. = post inoculation.

The results of this experiment show that acidification of the drinking water significantly reduced the transmission of *C. jejuni* between spatially separated animals. This finding is in line with earlier studies [8-10,18]. Furthermore we found that acidification of either the drinking water of sender animals or that of receiver animals or both is not significantly different. Moreover, we do find a significant negative interaction effect between acidification on the sender and on the receiver side. This indicates that the effect of acidification of the drinking water of both sender and receiver animals is not a multiplicative effect. A possible explanation arises from hypothesising selection of agent by acidification. When both inoculated and susceptible are acidified it is plausible that agent selection takes place at the inoculated (sender) side. Only agents capable of surviving an acidified environment (either inside or outside the host) will be able to get to the lower tracts of the intestine of the host and reproduce. Some evidence exists that *C. jejuni* has a mechanism of surviving in a stressful environment. For *C. jejuni* is known that the bacteria can go in a "dormant" state, called the viable but non-culturable state (VBNC) [19]. It has also been reported that these VBNC bacteria are able to return to a culturable state and cause an infection or colonisation [20]. When these (selected) agents are then secreted and transported to the susceptible animals (receivers) the acidified drinking water on this side might have less or no effect; resulting in the same transmission rate as found from acidification of either the sender or the receiver side.

The negative interaction effect indicates that it may be too simple to model indirect transmission probabilities as a product of probabilities of sub-processes. In particular the way in which the effect of intervention measures are represented in (mathematical) models needs to be considered carefully. Most between-farm transmission models do not consider the possibility of an interaction between different measures against (indirect) transmission [21,22]; instead transmission is modelled as a product of (decreased) probabilities. If there is indeed an interaction effect this may lead to an overestimation of the effect of interventions. This is dependent on whether the intervention causes a selection pressure on the pathogen, and whether the selection is fast enough to occur before the (selected) agent reaches new susceptibles (other farms); in those circumstances a control measure could have less effect than previously estimated. A recent and important example of this is the antibiotic resistance in bacteria.

As mentioned before the acidification of either drinking water or feed has been found to reduce pathogen transmission before in different studies. Therefore the results of this study are relevant too for other host-agent systems, in particular those where the faecal-route is the most important route of transmission. Van Gerwe *et al* estimated a transmission parameter (β) for direct *Campylobacter* transmission of 1.04 day^{-1} [23]. Comparing this with our estimate of 0.002 day^{-1} for indirect transmission, it is clear that indirect transmission is a less efficient process than direct transmission. This does not mean however that indirect transmission is less important epidemiologically. In fact, the spread of *C. jejuni* in the poultry industry is most probably a combination of indirect transmission for between-flock spread and direct transmission for within-flock spread. The estimates imply that the probability of introduction via indirect transmission into a susceptible flock is generally relatively low (i.e. there can be some delay in time before introduction occurs), once introduced however, *Campylobacter*

may typically spread very fast throughout a flock.

We observed a large variation in the number of colonised broilers between repetitions for the control group, as is shown in Table 1. There are three repetitions with a relatively low number of infections (repetitions 2, 3 & 4) and one repetition with a high number of infections (repetition 1). We chose, however, to pool the control repetitions for two reasons: first, we have previously found a significant effect of acidification of the drinking water [10], indicating that the repetition 1 is not a rare outlier. Second, unpublished data from four repetitions with normal tap water in a later experiment show two repetitions with the intermediate number of 4 infections, indicating that the current repetition 1 is not a very strong outlier.

To get more detailed insight in the role of sender and receiver in indirect transmission further experiments should be carried out. An interesting aspect is the effect of dosage of the pathogen on the colonisation both with and without acidification of the drinking water as this could provide additional information on the nature of the interaction effect.

Conclusion

In conclusion, we demonstrated that acidification of either the sender or the receiver side of the transmission chain has an effect on the indirect transmission of *C. jejuni* between broilers. We found that acidification of the drinking water has an effect on the transmission rate compared to a control situation with no acidified drinking water. However this effect is not multiplicative; there is no added advantage of acidifying both sides of the transmission chain.

Acknowledgement

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CHAPTER 4

Unexpected delay in transmission between spatially separated hosts

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Abstract

The lack of understanding of transmission between spatially separated hosts (indirect transmission) is an important knowledge gap in several disease control problems ranging from epidemics in livestock to outbreaks of hospital infections. In particular the mechanisms underlying this indirect transmission are little understood. In this study we carried out experiments on the transmission of *Campylobacter jejuni* between spatially separated broilers as a model to gain insight into the mechanisms of indirect transmission. The results showed an unexpected delay before the occurrence of the first transmission event. The delay pattern was unexpected as it was not consistent with a constant low overall transmission rate but rather it seemed that a first period with an extremely low rate transmission rate was followed by a period with a higher transmission rate. To explore possible mechanisms that could underlie the observed pattern, we extended the standard basic Susceptible-Infectious (SI) model to include the environment as an infectivity reservoir. Several scenarios were tested against the results of the transmission experiments. This revealed that the experimental observations can neither be explained by purely airborne transmission, nor by a simple, unstructured environmental reservoir. Instead, it is best described by a multistage environmental route from sending to receiving animal, suggesting that transmission occurs through progressive (but slow) contamination of the environment surrounding the source.

Introduction

Transmission between spatially separated hosts implies that no direct contact occurs between the infectious host (the source) and the susceptible host (the recipient). Hence the infectious agent travels via some route through the environment, a process commonly called indirect transmission. For example, for plant diseases indirect transmission is often well understood from the physics of fungal spores travelling from one plant to the next or to a neighbouring field [1]. Indirect transmission is also important for human and animal diseases, both in epidemic and endemic contexts. For example, a vast number of untraced infections commonly described as “neighbourhood” infections have been characteristic of recent major epidemics of classical swine fever, foot and mouth disease and highly pathogenic avian influenza in The Netherlands and other countries [2-6]. As the majority of these neighbourhood infections occurred after bans on animal movement had been implemented, this most probably indicates indirect transmission between farms. Similarly, indirect transmission is believed to cause a number of hospital infections. For example, it has been implicated in the spread of Methicillin-resistant bacterium *Staphylococcus aureus*, associated with hospitals (HA-MRSA). Transmission via (the hands of) health care workers or contaminated surfaces are thought to be important routes for these infections [7-9].

Some important efforts have been made to identify and quantify the possible routes of neighbourhood transmission of different livestock diseases [10-12]. However, knowing possible routes of transmission does not necessarily mean that the mechanisms and dynamics are understood. In particular the mechanisms are of major importance for implementing interventions and bio-security measures. Especially for the major epidemics that occurred in The Netherlands as described above indirect transmission is only poorly understood but also for other diseases [13, 14] it remains unclear what the role of indirect transmission is and what mechanisms underlie this form of transmission. More specifically we have no clear idea how the pathogens travel from sender to recipient and how much time they use. In presumed mechanisms for indirect transmission the time it takes for pathogen to travel is often short (for example transmission on fomites) or very short (airborne) but the probability of success is small.

Here, indirect transmission is studied in an experimental setting. Subsequently the experimental results are analysed using mathematical models in order to infer possible underlying mechanisms. The experimental setup consisted of inoculating naïve broilers with *Campylobacter jejuni* and monitoring the spread of *Campylobacter jejuni* from these source animals to spatially separated naïve recipient broilers. This experimental setup, serving as a model system for indirect transmission, was chosen for two reasons. First, indirect transmission has been observed to occur in a previous experiment with a similar setup [15]. Second, in the field, indirect transmission of *Campylobacter jejuni* occurs frequently between poultry houses and/or farms [16]. We use the mathematical models to investigate which possible transmission mechanisms can (best) describe quantitatively the experimentally observed transmission mechanisms. In the experiments we have chosen not to measure the presence of *Campylobacter* on surfaces in the environment between the inoculated and recipient broilers. The reason for this decision was that it was deemed impossible to sample

the environment sufficiently intensively for obtaining information useful for a quantitative interpretation without influencing the transmission mechanism(s). With regard to sampling the air, we note that detecting airborne *Campylobacter* in this experimental setup has been proven to be very difficult [17].

In order to analyse the experimental results we have developed, by extending the standard Susceptible-Infectious (SI)-model, a new class of models for indirect transmission [18-20]. From this class, different extended models were chosen, each representing a different hypothetical transmission scenario. Five biologically plausible scenarios were analysed to find out what mechanisms could underlie indirect transmission as observed in the experiments. In these analyses, the scenarios were tested against the experimental data, considering both quality of fit and biological plausibility of the fitted parameter values.

Materials and Methods

Ethics Statement

Animal experiments within the Animal Science Group (ASG) of Wageningen UR are performed in accordance with the provisions of the European Convention for the protection of vertebrate animals used for experimental and other scientific purposes (86/609 EG). In accordance with the Act on Experimental Animals of the Netherlands the use of experimental animals is granted to ASG by permit from the Dutch Government (licence DLO dd. 17 Feb. 2010. Licence nr. 40100). The protocol was approved by the Committee on the Ethics of Animal Experiments of the ASG.

Experimental design

Two separate experiments were carried out, each experiment consisted of two groups, adding up to four groups in total. In each group, five chicks were orally inoculated with a *Campylobacter jejuni* by gavage. The inoculated chicks (source animals) were housed together in one cage in the centre of an experiment room (a climate controlled room in an experiment facility). Ten chicks (recipient animals) were housed individually in cages surrounding this centre cage placed at approximately 80 cm (with a minimum distance of 75 cm and a maximum distance of 106 cm) (see Figure 1).

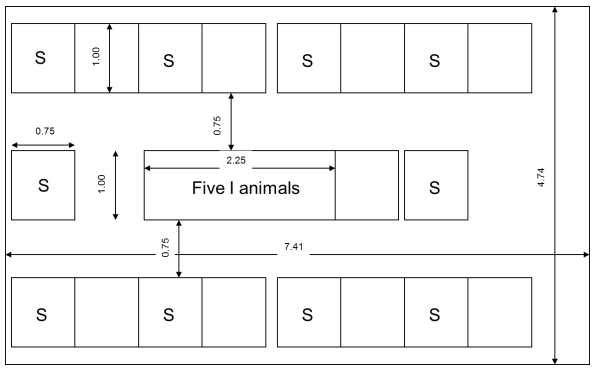


Figure 1. Schematic overview of the housing of the experimental groups. I = inoculated. S = susceptible.

To measure indirect transmission, all source and recipient animals were sampled daily by means of a cloacae swab (see section on Sampling). These swabs were tested within two hours after sampling in the laboratory for the presence of *Campylobacter jejuni*. If a tested recipient animal was found *Campylobacter jejuni* positive, the animal was considered infected and was immediately removed from the experiment to avoid having to deal in the analysis with multiple cages contributing to the infection pressure. The removed animals were euthanised and cecum was removed for further investigation for the presence of *Campylobacter jejuni*. The experiment ended 35 days post inoculation. All remaining source and recipient animals (that had not been found positive until that moment) were euthanised and cecum was removed and further investigated for the presence of *Campylobacter jejuni*.

Housing

From the day of arrival (day 0) until 12 days post-arrival, 60 broiler chicks were housed together in one group. On day 12, the chicks were equally divided over four experiment rooms for the transmission experiment. Each room contained five chicks (source animals) housed together in one centre cage and ten chicks (recipient animals) individually housed in ten cages surrounding the centre cage as shown in Figure 1. The distance from the centre cage to any one of the surrounding cages was at least 75 cm. All chicks were housed on wood shavings. The drinking water was supplied through a nipple drinking system. In each set-up, the drinking nipples in the cages on the long sides of the area were supplied from one common water container, while the centre cage had a separate drinking water supply. This precluded transmission via a shared drinking water system. Feed was given ad lib.; each cage had its own food supply. Before the start of the experiment, all experiment rooms were cleaned and disinfected with formaldehyde. Subsequently, samples were taken from 12 different areas inside the room to check for the absence of *Campylobacter jejuni*.

Animals

One-day old broilers (type Ross 305) were obtained from a commercial hatchery. On day 7 and day 12 after arrival, cloacal swabs taken from each chick confirmed the absence of *Campylobacter jejuni*.

Inoculation

For inoculation, the *Campylobacter jejuni* strain 356 [21] was used. The strain was freshly cultured in hearth infusion broth (microaerobically, 37°C, overnight) and diluted in buffered peptone water to obtain the intended inoculation dose (± $1{\cdot}10^6$ CFU/ml). The precise concentration (CFU/ml) of *Campylobacter jejuni* in the administered inoculum was determined by plating on modified cephoperazone charcoal deoxycholate agar (mCCDA) (Oxoid CM 793) with selective supplement (Oxoid CM 155) before and after the inoculation of the animals. Source animals were inoculated 14 days after arrival with 1 ml inoculum.

Sampling

To measure indirect transmission, all animals were tested by means of a cloacae swab.

After an inoculated chick (source animal) was found positive for *Campylobacter jejuni* on three consecutive days, swabs for those chicks were taken weekly instead of daily. For the susceptible chicks (recipient animals) swabs were taken once a day throughout the experiment. On days when both inoculated and recipient animals were to be sampled in each group, the recipient animals were sampled first. If a recipient animal tested positive for *Campylobacter jejuni*, the animal was immediately removed from the experiment and sacrificed for further investigation of the cecum. Samples were collected using sterile swabs (sterile plain dry swabs, Copan Diagnostics Inc., USA). Swabs were directly plated on mCCDA, incubated microaerobically at 41.5°C for 48 hours and examined for the presence of *Campylobacter jejuni*. The swab was then placed in Preston enrichment medium (Nutrient Broth no. 2, Oxoid CM0067 with *Campylobacter* selective supplement (Oxiod SR0204E) and *Campylobacter* growth supplement (Oxoid SR0232E)) and incubated microaerobically at 41.5°C for 24 hours. After incubation, it was plated on mCCDA and incubated microaerobically at 41.5°C and examined for the presence of *Campylobacter jejuni* after 24 and 48 hours.

Hygienic Measures

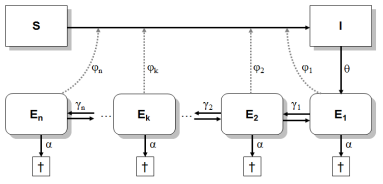
To prevent animal caretakers from acting as a vector of transmission, during the entire experiment strict hygienic measures were in place. Clean overalls were used at every entry into the experimental rooms. A pair of boots was dedicated to each room, cleaned on entering and exiting the room by means of wading through a chlorinated bath (Suma Tab D4, JohnsonDiversey). Sterile gloves were changed between handling individual animals. If both recipient and inoculated animals in one experiment room were to be sampled, the recipient animals were always sampled first, before the inoculated animals. Animals inside an experiment room were always sampled according to a fixed scheme. The different experiment rooms were also sampled according to a fixed scheme.

Statistical tests

To test for the presence of a temporal trend in the infection incidence the exact Cochran-Armitage test for trend was performed on the data. For this, the observation period was divided into five consecutive 7-day time intervals. In the test the expected number of infections in each time interval under the null hypothesis was calculated based on the number of susceptible present at the beginning of that interval and the infection probability in that interval according to the model to be tested.

Mathematical models

To describe indirect transmission we extended the standard SI (susceptible-infectious)-model to develop a general class of stochastic models [18-20]. The basis of this class of models is that susceptible (S) recipient animals may become infectious (I) by transmission of infectivity via one or more environmental reservoirs (E). These reservoirs have infectivity released into them either by infectious animals or by another environmental reservoir. All reservoirs are assumed to be completely clean at t=0, as is the case for the environment in the experiments. A schematic overview of this general class of models is depicted on the left panel of Figure 2;



Transition	Rate
$(S, I) \to (S-1, I+1)$	$\sum_{k=1}^{n} (\varphi_k E_k)$

$$\frac{\mathrm{d}E_k}{\mathrm{d}t} = \begin{cases} \theta I - \gamma_k E_k + \gamma_k E_{k+1} - \alpha_k E_k, & \text{if } k = 1 \\ \gamma_{k-1} E_{k-1} - \gamma_{k-1} E_k - \gamma_k E_k + \gamma_k E_{k+1} - \alpha_k E_k, & \text{if } 1 < k < \mathrm{n} \\ \gamma_{n-1} E_{n-1} - \gamma_{n-1} E_n - \alpha_n E_n, & \text{if } k = \mathrm{n} \end{cases}$$

Figure 2. Schematic flow diagram of the general class of models.

S = Susceptible, I = Infectious, E = Environmental reservoir. Greek letters denote rates. †=dead. Solid arrows indicate the direction of the flow from one compartment to another; the flow from S to I consists of animals; the flow from I to E_1 and to and from E-reservoirs consists of infectious material excreted by infectious animals; dotted arrows indicate (possible) influence of a reservoir on rate of transmission. Formally, the occurrence of a transmission event is denoted by a state change: the system moves from state (S,I) to (S-1,I+1), i.e. one animal moves from the susceptible compartment (S-1) into the infectious compartment (I+1).

the right panel shows the corresponding mathematical representation. A schematic overview of the four specific scenarios considered in detail below is shown in Figure 3.

Transmission events, indicated in Figure 2 by the arrow leaving the S compartment and pointing towards the I compartment, are modelled to occur according to a random (Markov) process with a rate determined by the force of infection. For indirect transmission (n>0), the force of infection is determined by the infectiousness of the environmental reservoir(s) indicated by the dashed arrows in Figure 2. From the I compartment there is a flow of infectious material into the E_1 compartment. This represents pathogen that the infectious animals shed into the environment. All models presented in this paper assume a constant shedding rate over the duration of the experiment (we will loosen this assumption in the model discussed in the Appendix) [22]. For this shedding process, as well as for transitions between different environmental compartments, we use a deterministic description, employing ordinary differential equations. The modelling of the environment by concatenated compartments can capture both the case of a multi-stage environmental route, as well as that of a stratified infectivity reservoir. An example of a multi-stage environmental route could be a spatial chain

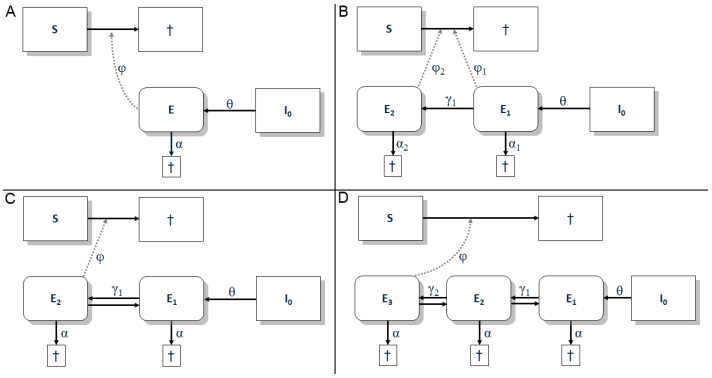


Figure 3. Schematic flow diagram of four scenarios.

A) 1-environmental reservoir. B) pathogen heterogeneity. C) 2-environmental reservoir. D) 3-environmental reservoir. S = Susceptible, I0 = Inoculated, i.e. at t=0, I0 is equivalent to I in Figure 2 (initial infected individuals, seeding transmission), E = Environmental reservoir. Greek letters denote rates. †=dead. Solid arrows indicate the direction of the flow from one compartment to another; the flow from S to I consists of animals; the flow from I to E consists of infectious material excreted by infectious animals; dotted arrows indicate (possible) influence of a reservoir on rate of transmission. Newly infected animals are removed immediately after detection of the infection, and hence have a negligible contribution to the infection pressure to the environment.

of transport events from source to recipient animal. An example of a stratified reservoir could be pathogen heterogeneity. If a multi-stage environmental route is modelled, only the last compartment is assumed to influence the transition from S to I (i.e. contributes to the force of infection). In a stratified reservoir all environmental reservoirs influence the transition from S to I. Finally, the model accounts for decay of the pathogen in the environmental reservoir(s), as depicted in Figure 2 by the solid arrows pointing downwards from the E-compartments. It can be shown mathematically (by rescaling the environmental variables E_i), that the model is equivalent to a model in which the shedding parameter θ only occurs as a factor multiplying the transmission parameter φ. As a consequence, θ will not be estimated separately; instead, it is sufficient to estimate the product of θ and φ.

We explored a number of specific scenarios from this general class of models for indirect transmission to identify models potentially able to explain the results of our experiments. We start with models with low numbers of estimable parameters, and add more complexity (only) if the simpler model fails to describe the observations satisfactorily; we aim to adopt the simplest model possible to describe indirect transmission, to reveal general principles regarding indirect transmission and assess the role of the environment. For the exact mathematical formulation of each of the models see Table 1.

The first scenario assumes that transmission is via the air; i.e. purely airborne transmission with no further environmental stage. Due to the short airborne journey time expected between the cages this scenario is described by the same model as that of direct transmission: the (standard) SI-model. The rational here being that even low wind speeds are of order of magnitude 0.1-0.8 m/s, implicating that travelling of the pathogen directly by air from sender to receiver would take seconds rather than days, effectively dropping the environmental reservoir from the model due to the difference in time-scales. This is the null-model, i.e. with no reservoirs (n=0). A second scenario extends the SI-model with a single environmental

Table 1. Stochastic infection rates used in the different model scenarios. N.A. = not applicable

Scenario	Rate of the transition (S,I) → (S-1,I+1)	ODE's of the deterministic part
1	βI	N.A.
2	φE	$\frac{dE}{dt} = \theta I_0 - \alpha E$
3	$\varphi_1 E_1 + \varphi_2 E_2$	$\frac{dE_1}{dt} = \theta I_0 - \alpha_1 E_1 - \gamma_1 E_1$ $\frac{dE_2}{dt} = \gamma_1 E_1 - \alpha_2 E_2$
4	φE_2	$\frac{dE_1}{dt} = \theta I_0 - \gamma_1 E_1 + \gamma_1 E_2 - \alpha E_1$ $\frac{dE_2}{dt} = \gamma_1 E_1 - \gamma_1 E_2 - \alpha E_2$
5	φE_3	$\frac{dE_1}{dt} = \theta I_0 - \gamma_1 E_1 + \gamma_1 E_2 - \alpha E_1$ $\frac{dE_2}{dt} = \gamma_1 E_1 - \gamma_1 E_2 - \gamma_2 E_2 + \gamma_2 E_3 - \alpha E_2$ $\frac{dE_3}{dt} = \gamma_2 E_2 - \gamma_2 E_3 - \alpha E_3$

infectivity reservoir (n=1). A schematic overview of the flow in this scenario, given in Figure 3 panel A, shows that it entails the transition from susceptible to infectious animal with a certain rate (φ), the excretion of pathogen in the (clean) environment by infectious animals with rate θ, and a decay of pathogen in the environment with rate α (death rate). This scenario assumes that there is no replication of the pathogen in the environment and therefore the death rate, α, cannot be negative. A third scenario hypothesises the presence of pathogen heterogeneity, which is captured in the model by dividing the pathogen population into two subpopulations as depicted in Figure 3, panel B. This could represent a situation in which a pathogen adopts a strategy to cope with a "hostile" environment by turning into a better surviving (but less infectious) state. The fourth and fifth scenarios consider the n=2 and n=3 cases of a multistage environmental route, as depicted in Figure 3, panel C and D. In biological terms, these could be interpreted as multistage routes, a series of spatial stages that have to be passed before the pathogen arrives at the (susceptible) recipient animal. These scenarios (4 and 5) allow for back-and-forth migration (i.e. a simplified diffusion process) between the environmental reservoirs to capture the spatial structure, and by assuming an "effective death rate" in the environmental reservoirs equal to the true death rate plus the rate at which the pathogen concentration thins (due to diffusion perpendicular to the path from sender to receiver) also the thinning effect due to the two-dimensionality of space can be approximately accounted for. The last two scenarios assume that the death rate of the pathogen is the same in all environmental reservoirs. Finally, we include as a sixth scenario a version of the model scenario 2 with a fixed (literature) value of the pathogen decay rate α in the environment.

Parameter estimation

For the estimation of the model parameters, a maximum-likelihood approach was used. Observations are made at discrete time points and thus the number of transmission events in the interval (Δt) has to be considered, which, to a good approximation, follows a binomial model with probability $p(t,t+\Delta t)=1-e^{-\int\lambda(t)dt}$, λ(t) being the infection hazard for a susceptible animal, and the number of animals still susceptible at time t as the binomial total. We identify the day before a recipient animal was found positive as the day of infection; this is motivated by experiments we carried out before [15] that show that there is virtually no delay between inoculation of animals with *Campylobacter jejuni* and detection of the bacteria in the faeces. This leads to the following expression for the model likelihood L:

$$L=\left(e^{-\varphi\int_0^T E(t)dt}\right)^{S_T}\cdot\prod_{i=1}^{M}e^{-\varphi\int_0^{t_{i-1}}E(t)dt}\cdot\left(1-e^{-\varphi\int_{t_{i-1}}^{t_i}E(t)dt}\right)$$

Here ST is the total number of susceptible animals escaping infection throughout the experiment, t_i is the day number (post inoculation (p.i.)) at which the i-th positive recipient animal was found positive, M is the total number of positive recipient animals, and T is the total number of days (p.i.).

The parameters where estimated by maximizing the likelihood using a numerical maximisation routine (NMaximize routine in Mathematica 7.0 [23])

We obtain the 95% confidence intervals for the maximum-likelihood estimates using the

likelihood ratio test; for each parameter univariate confidence bounds were calculated. For the calculation of the confidence interval for α in scenario 2 we used the score test because the point estimate for α is close to the boundary value of 0.

Assessing biological plausibility and model fit

We assess biological plausibility by comparing parameter estimates to what parameter values could be biologically expected. If the estimated parameter(s) for the given model is clearly outside the biologically plausible range, we reject the model. When obtaining several alternative non-rejected models, we determine their relative goodness-of-fit. As a measure for the relative goodness-of-fit of the models we used Akaike's Information Criterion (AIC) [24]. The AIC is calculated as $2k - 2\log(L_{max})$, where k is the number of estimated parameters in the model and L_{max} is the maximised value of the likelihood function for the model. The lowest AIC score indicates the (mathematically) preferred model. To compare different plausible models to each other we considered the ΔAIC score calculated as the difference with the model with the lowest AIC. We used the suggestions by Burnham and Anderson [24] for further selection among models: i.e. only ΔAIC > 2.0 indicate a possible difference in model fit between models. In addition, we constructed a QQ-plot [25] of the model distribution against the data. Such a plot facilitates a visual inspection of the quality of the model fit: the closer the data points are to the line y = x, the better the fit.

Results

Experimental results

All inoculated animals in all experiments were found positive within three days after inoculation. All inoculated animals remained positive throughout the experiment, and their ceca samples collected after finishing the experiment were also positive. Indirect transmission of *Campylobacter jejuni* occurred in three out of four experimental groups (Table 2). A total of 12 of the 40 recipient animals became infected at some point in time. The distribution of transmission events in time is shown in Figure 4. This figure shows a large delay before the majority of transmission events take place. After this initial delay, there is clustering of transmission events in time during the late stage of the experiment. We found no correlation between the spatial order of infection of recipient animals and the order of sampling of the animals, nor did we find a correlation between the order of infection and the air flow direction.

Modelling results

Five different scenarios were tested against the data. The results for all scenarios are

Table 2. Main results of the experiments: total number of infections and day number of infection per experiment room. (d.p.i. = days post inoculation).

Room	Total infections	Day number of infection (d.p.i.)
1	8	15,15,15,22,23,26,28,29
2	2	3,28
3	2	30,30
4	0	-

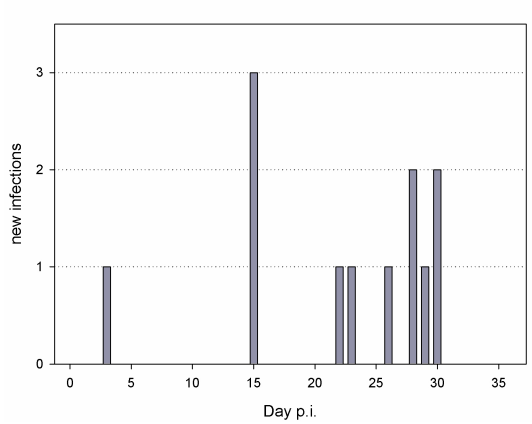


Figure 4. Experimental observed number of new infections per day after inoculation. (p.i. = post inoculation)

summarised in Table 3. The first model scenario, of direct airborne transmission, takes the form of the standard SI-model (n=0). With a constant number of infectious source animals (and thus a constant force of infection (we assume animals have a constant infectiousness over time)), this model predicts a constant rate of infection of the recipient animals. However, it is evident from Figure 4 and Table 2 that most transmission events happen in a late phase of the experiment, and the first events occur after a time delay. When tested statistically, correcting for decline in the number of animals remaining at risk through time, indeed a trend in the timing of infection (Cochran-Armitage test for trend, p=0.03) is found. This test in fact falsifies the n=0 model, showing that the delay cannot be simply explained by constant but low force of infection. This shows that the observed delay is unexpected under the direct airborne transmission scenario. The low quality of the model fit is illustrated visually by the model prediction plot in Figure 5 and by the QQ-plot (Figure 6).

The second model scenario (Figure 3, panel A, n=1) is able to reproduce the delay in transmission, as illustrated by the model prediction plot (Figure 5) and the QQ-plot (Figure 6). The model fit also has a lower AIC than for the first scenario (Table 3). However, when considering the parameter estimate for the decay rate α, we find that the model fit is not satisfactory biologically. The maximum-likelihood estimate of α is very close to zero; so close that we are only able to obtain a numerical upper bound: $0 \leq \alpha_{ML} < 5.6 \cdot 10^{-8}$. Such a low value for α corresponds to a survival time of the pathogen of many thousands of years; the upper confidence bound for α yields a minimum survival time of 15 days. However, as we know from literature, the survival time of *Campylobacter jejuni* is much shorter, for example Valdes-Dapena Vivanco & Adam reported a survival time of approximately 2 days, yielding α=0.5 [26]. On this basis, we reject this second model scenario as well. We have also fitted this model with α fixed at 0.5. The results of this model are in Table 3, scenario 6. Although the AIC of this model is higher than that of the first scenario the ΔAIC < 2, therefore, we cannot distinguish between these two models. The resulting model fit is poor however, as is evident from the model prediction plot (Figure 5) and the QQ-plot (Figure 6).

In the third scenario, of pathogen heterogeneity, for the first environmental compartment a

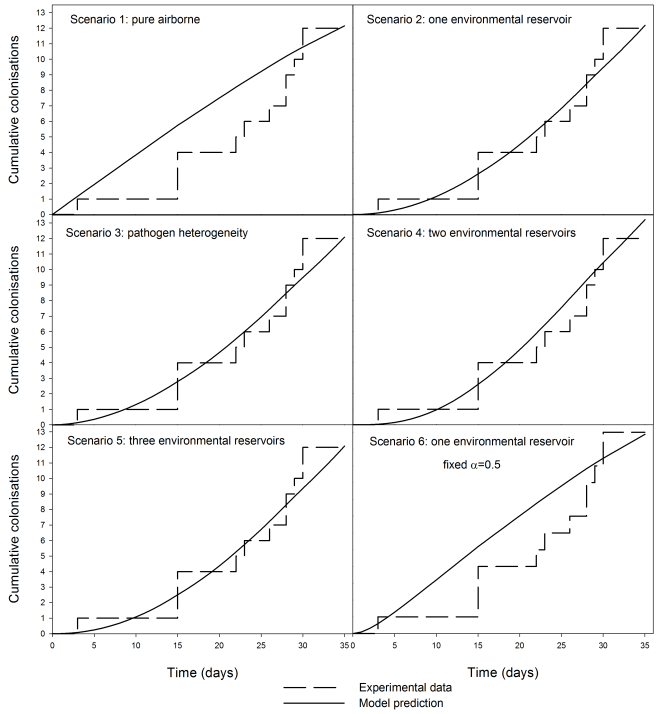


Figure 5. Model predictions of the different scenarios. These plots show the model predictions of cumulative infections in time (solid line) against the experimentally observed cumulative infections (intermittent line).

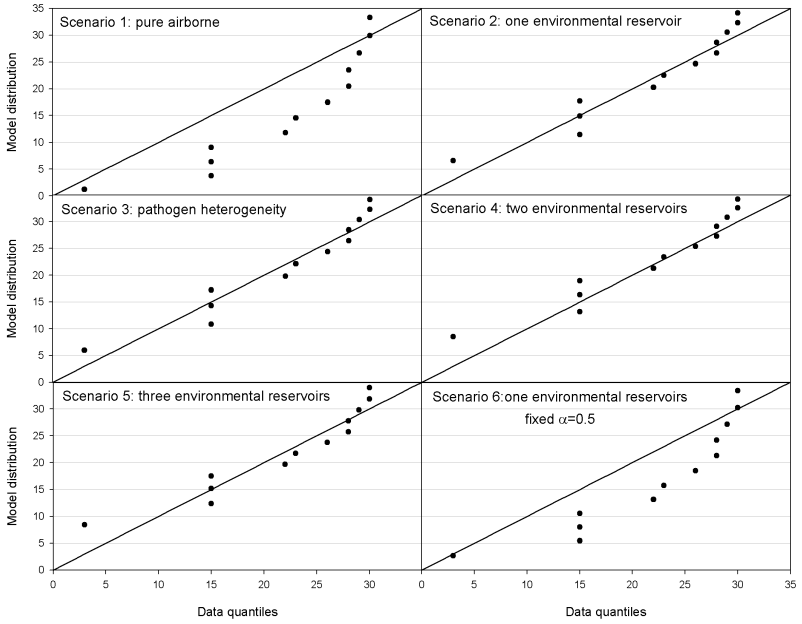


Figure 6. QQ-plots of the different scenarios. These plots show the model distribution of infections in time against the experimentally observed distribution of infections.

fixed literature value for α (0.5) was used (Figure 2, panel B). The values for the remaining parameters were again estimated using a maximum-likelihood approach. This produced a very high estimated value of the exit rate γ ($2.9 \cdot 10^3$). This means that there is virtually no pathogen in the first environmental reservoir, i.e. effectively the model behaves the same as the n=1 model of scenario 2 and therefore does not describe a scenario of pathogen heterogeneity. Consequently this model was also rejected.

The fourth scenario, in which the pathogen has to "travel" through two environmental reservoirs before reaching the susceptible host (Figure 3, panel C), is the first that produces a satisfactory fit to the data. The parameter estimation yields biologically plausible values (Table 3, scenario 4). Accordingly, with the assumed literature value for α, the model can reproduce the observed delay in transmission, as is visualised by the model prediction plot in Figure 5 and in the QQ-plots in Figure 6.

In the fifth and last scenario (Figure 3, panel D) we also obtain biologically plausible values (Table 3, scenario 5). However, the AIC is higher than in scenario 4 and the difference in AIC compared with scenario 4 is more than 2, indicating a relevant difference in model fit. Thus, the additional complexity of the extra reservoir in scenario 5 is not producing sufficient improvement in model accuracy. Figure 7 shows the infectivity of the different environmental reservoirs from scenario 4 (top row) and scenario 5 (bottom row). From these figures we see that the second and third environmental reservoir of scenario 5 show an almost indistinguishable dynamics.

Table 3. Parameter estimates for the different model scenarios and relative goodness-of-fit.

Scenario	Parameter	Biological Description	Estimate [95% C.I.]	AIC	ΔAIC
1	φ	Transmission parameter	0.01 day^{-1} [0.0053 – 0.016]	136.98	0
2	α	Death rate	$5.6 \cdot 10\text{-}8$ day^{-1} [0 - 0.066]	134.30	n.a.†
	θφ	Combined shedding rate and indirect transmission rate	0.0005 day^{-2} [0.00032-0.00636]		
3	α	Death rate of 2nd reservoir	0.088 day^{-1} [$1.8 \cdot 10^{-8}$ – 0.611]	138.32	1.34
	γ	Transfer rate	$2.9 \cdot 103$ day^{-1} [96.33 - $2.1 \cdot 10^7$]		
	$\theta\varphi_2$	Indirect transmission rate of 2nd reservoir	0.0005 day^{-2} [0.00007 - 0.0058]		
4	α	Death rate	0.1 day^{-1} [$1.2 \cdot 10^{-6}$ - 4.4]	137.02	0.04
	γ	Transfer rate	0.0001 day^{-1} [$1.5 \cdot 10^{-8}$ – 6.9]		
	θφ	Combined shedding rate and indirect transmission rate	0.06 day^{-2} [$2.0 \cdot 10^{-5}$ – 6.9]		
5	α	Death rate	0.21 day^{-1} [$1.4 \cdot 10^{-7}$-6.5]	139.83	2.85
	γ_1	Transfer rate from 1st to 2nd reservoir and back	0.01 day^{-1} §		
	γ_2	Transfer rate from 2nd to 3rd reservoir and back	6.29 day^{-1} §		
	θφ	Combined shedding rate and indirect transmission rate	0.007 day^{-2} §		
6	θφ	Combined shedding rate and indirect transmission rate	0.0051 day^{-2} [0.003-0.009]	138.31	1.33

In the last columns the Akaike Information Criterion (AIC) and the difference between the model with the lowest AIC score and the other models is given. See text for the calculation of the AIC and ΔAIC. Scenario 1=direct transmission, 2=one environmental reservoir, 3=pathogen heterogeneity, 4=two environmental reservoirs and 5=three environmental reservoirs, 6=one environmental reservoir, fixed α.

†: biologically implausible estimated parameter values, therefore this scenario was excluded from ΔAIC calculations.

§: Estimation of the 95% confidence interval is not possible due to numerical instability of the system.

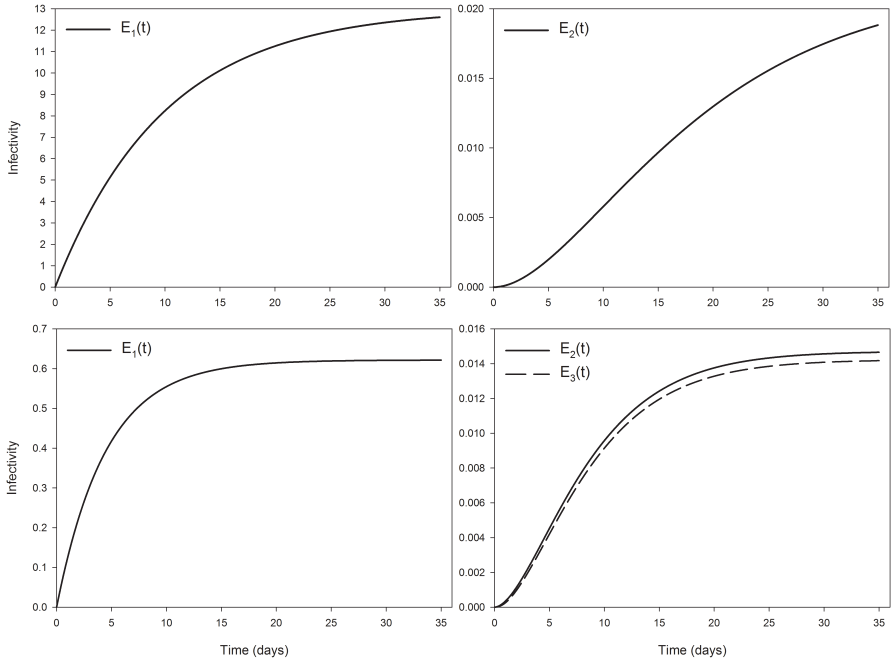


Figure 7. Model prediction for the infectivity of the environmental reservoirs from scenario 4 (upper two graphs) and scenario 5 (lower two graphs) as a function of time.

Discussion

This study considered the mechanisms of indirect transmission by carrying out experiments on the transmission of *Campylobacter jejuni* between spatially separated broilers and by using mathematical modelling to test mechanistic scenarios against the experimental observations. We observed a delay in the onset of indirect transmission, after which the risk of recipient animals becoming infected increased significantly towards the end of the experimental period (Table 2 and Figure 4). By mathematical modelling, we have shown that these findings imply that the possibility of "purely" airborne transmission can be ruled out as an explanation of the observations, although we can explain the delay with a low constant rate of infection, the clustering of transmission events late in time however cannot be explained by the low constant rate.

To identify mechanisms that could underlie the observed transmission pattern, we have proposed a general class of models for indirect transmission. Model scenarios assuming a single environmental infectivity reservoir or assuming pathogen heterogeneity (Table 3, scenarios 2 & 3) were found to yield parameter estimates inconsistent with known survival properties of *Campylobacter jejuni*. However, scenarios in which two or three linked environmental reservoirs have to be crossed by the pathogen to travel from the infectious host to the susceptible host (Table 3, scenarios 4 & 5), did yield a consistent explanation for the observed transmission pattern with biologically plausible parameter values. The linked reservoirs could be interpreted as the path travelled by the pathogen from an infectious host

to a susceptible host. We do note that for scenario 4 the transfer rate γ seems to be quite low, 0.0001 day^{-1}, meaning that on average a particle would need 10000 days (≈30 years) to travel between the two environmental reservoirs. However this figure can also be interpreted as that only 1 particle per 100 hundred particles is being moved every, on average, 10 days. Furthermore, the large confidence bounds found for a number of parameters give little or no biological information about the actual (biological) value of the parameter; however, as mentioned in the introduction, measurements in the environment has been proven to be extremely difficult [27], therefore we explicitly use the observed transmission patterns to infer possible mechanisms of transmission instead of directly measuring possible routes in the environment.

A large difference in total numbers of animals infected was observed between one experiment room and the three others. It is possible to incorporate variation between rooms in the model analyses, but this does not alter the results (data not shown), showing that the model outcomes are robust. Furthermore, the observed delay is present both in experiment rooms with low numbers of infection and in those with high numbers of infections.

In the experimental setup there was a slight variation in the distance between individual cages from the centre cage (75-106 cm) as depicted in Figure 1. These differences in distances did not yield a measurable distance effect; cages closer to the centre cage were not infected earlier than cages further away.

One relevant scenario that is not captured by the general class of models is a time dependency of the excretion parameter θ, i.e. a changing infectiousness over time. Such a time dependency could arise for example when the shedding of infectivity increases with the age of the source animal. Such an increase, for pathogens that colonise the intestinal tract, could arise from an increase in the amount of faeces excreted. We found that this scenario shows a less good model fit than the best-fitting scenario discussed above (two-compartment multistage model, scenario 4). The complete results of this extension to the general class of models are given in the Appendix; as our model analyses indicate, the rate of pathogen decay in the environment is a very important determinant of the predicted transmission pattern. As a result, if the environment is more favourable for pathogen survival than in the system studied here, different indirect transmission mechanisms might become possible for which other scenarios (or sub-models) from our class of models might yield a better description. For example, in the case of an aquatic environment that is visited by migratory birds in consecutive seasons, as in Breban *et al* and Rohani *et al*'s study of yearly transmission of low pathogenic avian influenza through an aquatic environment [28-31], it may be sufficient to incorporate just one environmental compartment [32-34].

In conclusion, we found that transmission of *Campylobacter jejuni* can occur between spatially separated hosts and that there is a significant delay of the transmission events. This delay is unanticipated, when assuming a pure airborne transmission scenario. Indeed, such an air-borne scenario is shown to be falsified by our observations. As we have shown, however, these observations can be explained by a number of alternative scenarios. The scenario that best explains the data is a multi-staged route from source to recipient.

These findings yield insight into the possible mechanisms of indirect transmission of pathogens

and can be of importance for developing better control measures against such transmission. In particular, the class of mathematical models developed may serve as a conceptual framework for analysing indirect transmission in the field. With respect to control measures, if indirect transmission is shown to take place via consecutive contamination of different surfaces (environments), breaking one of the links in the chain (e.g. by decontamination) may be either sufficient to break the whole chain or it at least decrease indirect transmission, thereby decreasing the risk of transmission.

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Appendix

Here we extended the general class by assuming a time-dependent shedding parameter θ(t). This parameter is assumed to increase linearly in time, which is interpreted biologically as a growth dependent excretion rate of pathogen into the environment (growth hypothesis).
The equation describing the shedding into the environment in this model is:

$$\frac{dE}{dt} = \theta(t) I_0 - \alpha E$$

Where $\theta(t) = \theta_0 + \theta_1 \cdot t$ and θ_1 is estimated from growth data.
The parameter φ describes the infectivity of the environment, θ(t) describes the shedding rate of the infectious hosts and α describes the death rate of the pathogen in the environment. The function $\theta_0 + \theta_1 \cdot t$ describes the linear relation of pathogen excretion with time. Parameter θ_1 was estimated from weight data of the experiments by means of linear regression and then scaled to yield a maximum of 1 after 35 days post inoculation.
Table S1 lists the parameter estimates. The AIC for this model is 138.84. The resulting QQ-plot, given the parameter values corresponding to the maximum likelihood, is given in figure S1.

Table S1. Estimates for the parameters.

Parameter	Estimate
α	0.176
θ_1	0.033
φ	0.126

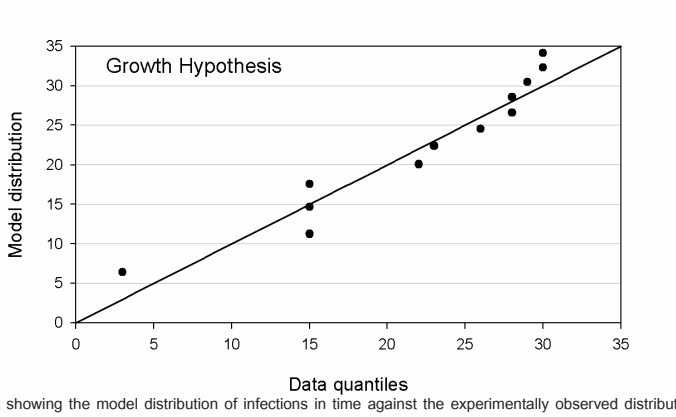

Figure S1. QQ-plot showing the model distribution of infections in time against the experimentally observed distribution for the growth hypothesis model.

CHAPTER 5

Small distances can keep bacteria at bay for days

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Abstract

Transmission of pathogens between spatially separated hosts, i.e., indirect transmission, is a commonly encountered phenomenon important for epidemic pathogen spread. The routes of indirect transmission often remain untraced, making it difficult to develop control strategies. Here we used a tailor-made design to study indirect transmission experimentally, using two different zoonotic bacteria in broilers. Previous experiments using a single bacterial species yielded a delay in the onset of transmission, which we hypothesised to result from the interplay between diffusive motion of infectious material and decay of infectivity in the environment. Indeed, a mathematical model of diffusive pathogen transfer predicts a delay in transmission that depends both on the distance between hosts and on the magnitude of the pathogen decay rate. Our experiments, carried out with two bacterial species with very different decay rates in the environment, confirm the difference in transmission delay predicted by the model. These results imply that for control of an infectious agent, the time between the distant exposure and the infection event is important. To illustrate how this can work we analysed data observed on the spread of vancomycin-resistant *Enterococcus* in an intensive care unit. Indeed, a delayed vancomycin-resistant *Enterococcus* transmission component was identified in these data, and this component disappeared in a study period in which the environment was thoroughly cleaned. Therefore, we suggest that the impact of control strategies against indirect transmission can be assessed using our model by estimating the control measures' effects on the diffusion coefficient and the pathogen decay rate.

Introduction

Indirect transmission, i.e., transmission without direct contact between hosts, is a ubiquitous mechanism of disease spread in epidemics as has been demonstrated in plants (e.g., refs. 1–3), in livestock (e.g., refs. 4–8), and in humans (e.g., refs. 9–12). Indirect transmission is important because, although control measures can prevent direct contacts, it is unclear how indirect contacts can best be avoided. For example, indirect transmission in health care facilities is believed to be the underlying mechanisms for a number of hospital infections, and as such has been implicated for example in the spread of methicillin-resistant bacterium *Staphylococcus aureus* associated with hospitals. Transmission via (the hands of) health care workers or contaminated surfaces are thought to be important routes for these infections [9–11]. Similarly, in the experimental study of the airborne transmission of *Bordetella pertussis* [12], it was found that there can be pathogen transmission without physical contact and that distance between separately housed animals plays an important role in determining whether naïve animals can actually get infected and the time it will take for infection to happen. Although highly important, knowledge of the possible routes of transmission alone is often insufficient to understand the mechanisms and dynamics of the disease transmission. A better understanding of the mechanisms that underlie indirect transmission is needed to improve effectiveness of biosecurity measures to control disease spread.

Here we obtain mechanistic insight by studying indirect transmission in controlled experiments and by using mathematical modeling to understand the experimentally observed transmission patterns. In previous experiments where a single bacterium species, *Campylobacter jejuni* (*C. jejuni*), was used, a delay in the onset of the first transmission events was observed when there is a (small) distance between colonised animals and recipients; however, when birds are in direct contact this delay is not observed, showing that the early pathogen excretion is sufficient to cause infection [13, 14]. These observations have led to the hypothesis that the observed delay is the result of a combination of diffusive movement of pathogen in the environment and decay of this pathogen while traveling from colonised animals to recipient animals. To test this hypothesis, tailor-made experiments were carried out, in which we concurrently inoculated broilers with two different pathogens with very different decay rates in the environment, namely *C. jejuni* and *Escherichia coli* (*E. coli*), and then studied the indirect transmission of these pathogens to spatially separated susceptible recipients.

In the mathematical model, we assume that pathogen-containing particles are randomly displaced through the environment according to a two-dimensional (2D) diffusion process, and we account for the decay of pathogen during its transit time in the environment.

Materials and methods

Analyses of the Experimental Data.

The experimental setup consisted of, in each replicate, inoculated infectious broilers in a center cage surrounded by 10 recipient broilers placed individually in cages at a distance of ~75 cm both from the center cage and from each other (Figure 1). All broilers in the center cage were inoculated with either *C. jejuni* or both *C. jejuni* and a labeled *E. coli* (see Table S1 for inoculation scheme). Both being commensal organisms to broilers, we expect no

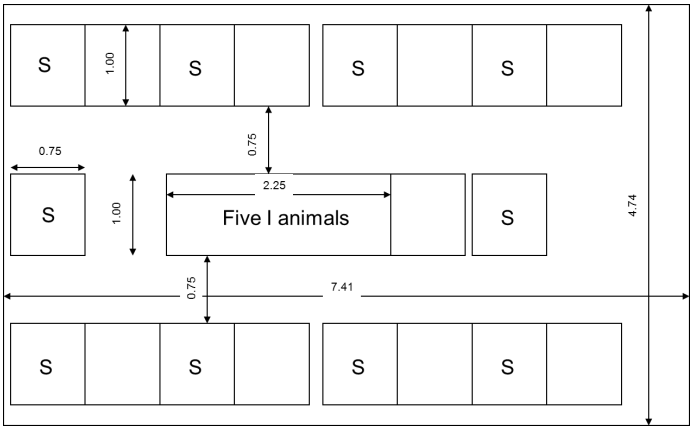

Figure 1. Schematic overview of the housing of the experimental groups of 5 or 20 inoculated animals in a center cage and 10 susceptible recipient animals in individual surrounding cages. Alongside the arrows, distances are given in meters.

important interference between the two species, and comparison of the data for *C. jejuni* only replicates and those with both *C. jejuni* and *E. coli* show no signs of interference in terms of colonisation times (Student t-test: $p = 0.27$ for the group with 5 inoculated animals and $p = 0.31$ for the group with 20 inoculated animals). The occurrence of indirect transmission events was monitored by a daily collection of cloaca swab samples from all recipient broilers. The experiment ended 35 d post inoculation (p.i.) (see Appendix for full description of experiment). In mathematical models, direct pathogen transmission is usually assumed to occur instantaneously when susceptible and infectious individuals are at the same location at the same time [17–19]. Modeling indirect transmission necessitates inclusion of the transport of infectious material in the environment between hosts, thereby allowing for time delays between pathogen shedding by an infectious host and subsequent exposure of a recipient host [20, 21]. To quantify the indirect infection pressure experienced by a susceptible recipient at a specific location at a specific time, the full history of how many infectious individuals were present at particular locations up until the time of interest needs to be taken into account. Here we developed a model in which the transport process was assumed to be diffusion of particles, i.e., infectious material was assumed to move with small random steps [22, 23]. One appealing consequence of this simplification is that we do not have to parameterise unobserved individual displacements of infectious material through the environment. Instead, we fit a single parameter (the diffusion coefficient) to the observed pattern, averaging over all transport routes. We assume that the diffusion of both *C. jejuni* and *E. coli* through the environment is governed by one and the same diffusion coefficient. This is motivated by the fact that both *C. jejuni* and *E. coli* are transmitted fecal–orally, thus, both pathogens are most probably transported on the same material. Moreover, in this case the two bacteria were excreted by the same animals. Cages with infectious broilers are modeled as an area source of pathogen-containing particles from which diffusion at rate D to the recipient cages occurs. For an area source emitting with strength Q_0 during a time interval $[0, \tau]$, the concentration of viable infectious material at a given location (x, y) at time t is obtained by integrating the

point-source solution of the diffusion equation over both space and time taking into account the decay rate (α):

$$S_{cont}(x,y,t)=\int_0^{\tau}\int_{y_1}^{y_2}\int_{x_1}^{x_2}\frac{Q_0}{4\pi D(t-t')}\exp\left[-\alpha(t-t')-\frac{(x-x')^2+(y-y')^2}{4D(t-t')}\right]dx'dy'dt'$$

The force of infection (FOI) experienced by a recipient animal is assumed to be proportional to the average concentration across its cage floor area. However, this is true for as long as the concentration is (much) smaller than an "exposure capacity" K [24]. For larger concentrations, the FOI is assumed to be bounded by a maximum equal to βK (with β being the transmission parameter, see below and Appendix), which is determined, for instance, by limitations in access to and/or uptake of infectious material by recipient animals. This formulation ensures that, even in the limit of negligible pathogen decay, the infection rate will remain within biologically plausible bounds. See Appendix for the resulting equation. The model parameters and their dimensions are listed in Table 1. The parameters that need to be estimated from experimental observations are the diffusion coefficient D, the transmission parameter β_{campy} for *C. jejuni*, β_{coli} for *E. coli*, the exposure capacity K and the decay rates of the pathogens β_{campy} and β_{coli}. The two decay rates are estimated independently from the transmission experiments in separate survival experiments (see Appendix for full description of experiments), carried out under the same conditions as the transmission experiments. Estimated decay rates were 2.25 d^{-1} for *C. jejuni* and we used 0 for *E. coli*, as we observed 100% survival during more than 100 d (see Table S2). The remaining parameters were estimated using a maximum likelihood estimation approach (see Appendix for the derivation of the likelihood equation).

Analysis of the ICU Data.

The data of Hayden *et al* [15], on the spread of vancomycin-resistant Enterococcus (VRE) in an intensive care unit (ICU), were reanalysed in this study to evaluate if the observed pattern of transmission provides evidence for a delayed/diffusive transmission component. A detailed description of the setup of this study can be found in the original paper.

In brief, the original study was intended to assess the performance of three different intervention schemes on the spread of VRE. It comprised of four study periods, each with

Table 1. Dimension and description of parameters used in the model.

Parameter	Dimension	Description
S_{cont}	$\#/m^2$	Concentration of pathogen on the time and location of interest
t`	day	Time of release of the particles
t, τ	day	Time of interest
(x`, y`)	(m,m)	Location in the source cage
(x,y)	(m,m)	Location in the recipient cage
x1, x2, y1, y2	m	Coordinates of the source cage corners
xa, xb, ya, yb	m	Coordinates of the recipient cage corners
D	m^2/day	Diffusion coefficient
α	day^{-1}	Decay rate of the pathogen
K	$\#/m^2$	Exposure capacity
β	day^{-1}	Transmission parameter

different (sets of) interventions: a baseline period (baseline, period 1); a period with intensified environmental cleaning (treatment 1, period 2); a "washout" period without any specific intervention (treatment 2, period 3); and a period with multimodal hand hygiene (treatment 3, period 4). During the study period, rectal swab samples were taken daily from patients starting on the day of admission throughout the admission period. Cultures for VRE were performed of those swabs.

Improved environmental cleaning (treatment 1, period 2) involved explaining to housekeepers the importance of environmental cleaning and increased monitoring of housekeeper performance in addition to the actual environmental cleaning. It also involved daily cleaning of ventilator control panels as well as sensitising nurses and other ICU staff about the problem of VRE and the interventions.

There were a total of 21 ICU beds available for admission of patients throughout the study period. In total, 748 admissions to the ICU were studied and the average duration of stay was not significantly different for the four periods. Using this data, the daily infection rate per person after being admitted to the ICU was calculated as a function of days postadmission. Differences between rates of colonisation for two window periods were analysed using a Fisher's exact test with the level of significance set at $p < 0.05$.

Results

The observations of our transmission experiments are summarised in Figure 2. A key observation was the difference in timing of the first transmission event for the two pathogens (Figure 2). For *E. coli*, there is a delay of 4 d post inoculation (p.i.) to the first transmission

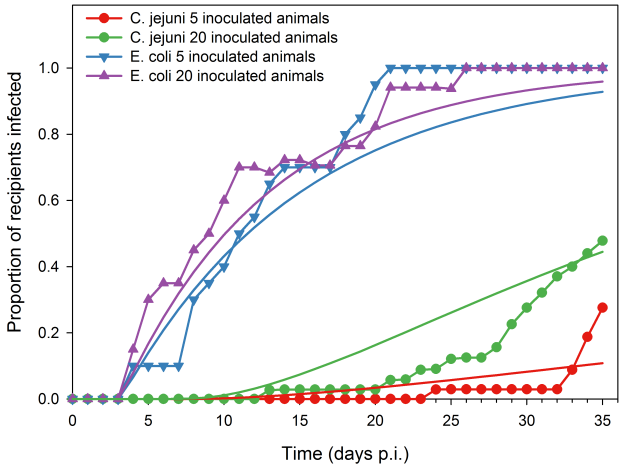

Figure 2. Proportion of recipient animals infected with *C. jejuni* or *E. coli* as function of time since inoculation of the inoculated animals. In the transmission experiment each experimental room contained 5 or 20 inoculated animals that were inoculated with either *C. jejuni* or with both *C. jejuni* and *E. coli* and 10 susceptible recipient animals. Curves with circles depict the animals that were infected through indirect transmission with *C. jejuni*. Curves with triangles depict the animals that were infected through indirect transmission with *E. coli*. Solid lines without symbols depict model predictions for that specific treatment. For *C. jejuni* the curves represent the proportion infected of the total number of recipient animals. For *E. coli* the curves represent the proportion infected of those recipient animals still present on that day, thus correcting for recipient animals that are infected with *C. jejuni* and were removed (See Appendix).

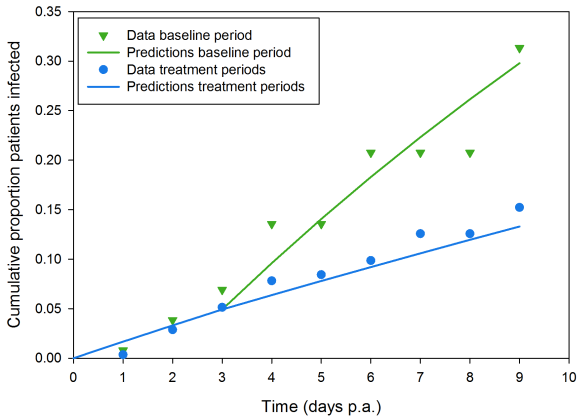

Figure 3. Cumulative proportion of patients infected per day after intensive are admission for the baseline period and all periods combined. Day 0 is the day of admission. Baseline refers to the period with no intervention, treatment periods refers to the treatment periods in which intensified cleaning was carried out (see Materials and Methods for further details). Note that only from day 4 onward, the slope of the baseline period is different from the treatment periods.

event for both the groups with 5 and those with 20 inoculated animals. For *C. jejuni*, the first transmission events occurred on day 12 p.i. for the groups with 20 and on day 23 p.i. for those with 5 inoculated animals. The large difference in observed delay to transmission onset between *C. jejuni* and *E. coli* is in complete accordance with the prediction of our mathematical model description of the hypothesised diffusive transport of infectious material with decaying infectivity (see Materials and Methods for the model description). In Figure 2, a parameter fit of this diffusion model for indirect transmission (solid lines without symbols) to the data explains in detail the difference in the onset of transmission of the two pathogens. The corresponding estimates of the parameters are listed in Table 2.

To illustrate how delayed transmission from a distant source could be affected by cleaning of the environment a different analysis of the ICU data of ref. 15 was carried out (Materials and Methods). In newly admitted patients we found a delayed component in the rate of VRE acquisition (with a delay of 4 d) in a period without intensified cleaning (Fisher's exact test, $p = 0.038$, Table 3). This delayed transmission component is not observed in the period with intensified cleaning (Table 3 and Figure S1). Figure 3 shows the cumulative relative

Table 2. Estimated values and 95% confidence intervals (CI) for the model parameters.

Parameter	Point estimate (95% CI)	
	5 I-animals	20 I-animals
D	0.003 (0.002 - 0.004)	0.0025 (0.002 - 0.005)
$\beta_{campy}=\beta_c$	0.007 (0.004 - 0.015)	0.015 (0.0053 - 0.0196)
$\beta_{coli}=\beta_e$	0.023 (0.0145 - 0.0345)	0.025 (0.016 - 0.037)
K	$1\cdot10^{-15}$ ($9.6\cdot10^{-20}$ - …)†	$1\cdot10^{-15}$ ($3.5\cdot10^{-21}$ - …)†
α_{campy}	2.25	2.25
α_{coli}	0.0	0.0

I-animals: inoculated animals.

†Estimation of the upper bound is not possible due to numerical instability of the system for large K.

Table 3. Average colonization rate per period for the baseline situation and the three treatments of the ICU transmission data.

Treatment	Period 1 (day 1-3 p.a.)	Period 2 (≥ day 4 p.a.)	p-value
Baseline	0.023495	0.050085	0.038
Treatment 1	0.021336	0.003527	0.210
Treatment 2	0.014849	0.014844	0.631
Treatment 3	0.015618	0.010426	0.532

A p-value < 0.05 indicates a significant difference between the colonization rate in period 1 and period 2. p.a. = post admission

number of infected patients against time post-admission for the baseline period and all other (treatment) periods together.

Discussion

The combination of animal experiments and modeling carried out here provides insights into the possible mechanisms underlying disease transmission as well as possibilities to quantify effectiveness of infection control measures. The model developed contains a single parameter, namely the diffusion coefficient D, which describes how the pathogen travels on its transport medium (i.e., excreta and/or dust) and which is displaced by external disturbances (e.g., by wind, humans, animals, and machines). In our experiments, these disturbances may include but are not limited to: actions by the animal caretakers; airflow due to the ventilation system; and/or behavioral actions such as wing flapping by the broilers themselves. Other unobserved external disturbances might also have taken place, further enhancing the pathogen-diffusion process. In this model, the diffusion coefficient D is the natural parameter for assessing the role of biosecurity measures in limiting pathogen displacement. For instance, experiments with safe model microorganisms (e.g. live vaccines) could be performed to compare estimated values of D with and without interventions. We note that the value of D is expected to depend on the type of material on which the pathogen is diffusing in the environment. If different microorganisms are transmitted concurrently in the same way (e.g. fecal-orally as in our case), then D can be assumed to be independent of the pathogen type. As the two bacteria used in our experimental setup are excreted in similar amounts and by the same animals, our model fit explains the difference in timing of first infection events in terms of the difference in pathogen decay during transit from source to recipient. This difference between *C. jejuni* and *E. coli*, in the predicted delay until the amount of infectious material available to recipient animals becomes sufficient to cause infection, is further illustrated by Figure 4. For any given time, the force of infection is higher for the groups with 20 inoculated animals compared with 5 inoculated animals, but the difference in delays is maintained. Survival experiments described in the Appendix show that *E. coli* bacteria survive almost the entire experimental period and *C. jejuni* bacteria only survive for on average 0.44 d ($\alpha = 2.25\ d^{-1}$). As a result, the accumulation of pathogens in the environment is much slower for *C. jejuni* compared with *E. coli*. At a given location and for a steady emission of pathogens from colonised/infected sources, the model predicts a saturation level of pathogen accumulation. That level is determined by the time needed to reach a location and the decay occurring during that time. This level is predicted to be lower for *C. jejuni* compared with *E. coli*. In addition, the model predicts that there is a limit to the distance that pathogens can reach in substantial amounts. Formulated more precisely,

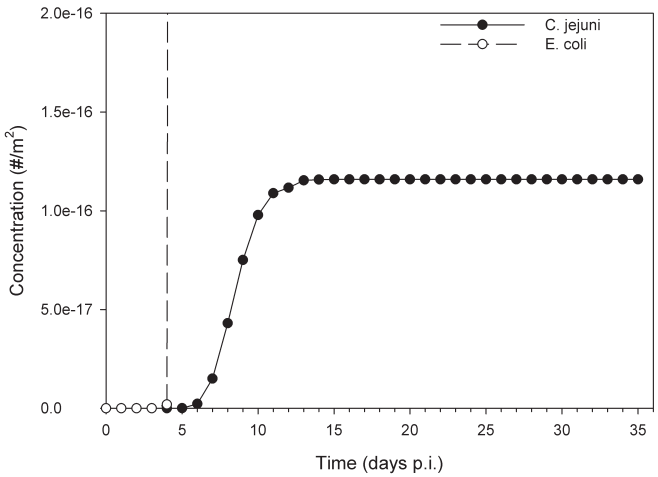

Figure 4. Prediction of the amount of infectious material per unit area in a recipient cage as a function of time. Note that the curve for *E. coli* quickly rises beyond the scale of this graph because, for *E. coli*, a decay rate value of 0.0 d^{-1} was used. Open circles depict the amount of viable *E. coli*. Closed circles depict the amount of viable *C. jejuni*. For the construction of the figure, the center cage (Fig. 1) was taken as the area source and a cage alongside the center cage as the recipient source. Parameter values used: D = 0.0025 m^2/d, α_{campy} = 2.25 d^{-1}, α_{coli} = 0 d^{-1}.

for every pathogen quantity level there is a maximum distance at which that quantity level can be realized (Figure S2). This distance limit is determined by the decay rate and the diffusion coefficient D.

The data (Figure 2) clearly show that there is a delay between the excretion by the senders and the infection of the recipients. We argue that the delay occurs during the travel/transfer of infectious material from sender to recipient, as separate observations imply the absence of a delay on either the sender or the recipient side: As was shown in direct transmission experiments using *C. jejuni* [13], the sender is excreting the bacteria from very soon after inoculation onward and these bacteria are immediately able to infect direct-contact recipients. Furthermore, it was observed in other previous experiments [14] that the recipients showed excretion always within one or two days after inoculation.

Therefore, the delay has to occur during the travel/transfer of infectious material from sender to recipient. Modeling the travel as a random walk, i.e., diffusion in 2D space (and including the decay of the pathogen while traveling), has the advantage that it explains both the delay and the difference in delay between the two bacterium species. Modeling it only as a buildup in the environment without a spatial component [16] would not explain the difference in delay. Although in our current model the diffusion is precisely following a 2D diffusion equation, this might not necessarily be the case; the movement of the infectious material in space does not have to be a random walk as we assumed. However, we believe that the random walk is a sensible model to start with as it needs no additional information.

Furthermore, the model predicts that infections with microorganisms having low decay rates can occur at distant locations (long) after the source of infectious material has been removed. This would have important consequences in, for example, hospital ICUs where this would imply that removing (or quarantining) a patient colonised with a certain pathogen might not

prevent subsequent transmission if that pathogen survives well in the environment. As an example of this, we investigated data from a study of VRE in an ICU [15]. In the original study, intensified environmental cleaning was associated with reduced acquisition of VRE. Given that newly admitted patients enter the ICU in a clean (and sterilised) bed, we assume that the surfaces immediately surrounding such a patient are initially not contaminated with VRE. However, without sufficient cleaning, the farther inanimate environment of a patient may still be contaminated with VRE from patients previously occupying the unit. Indeed our analysis of the ICU data shows the presence of a delayed transmission component, with a delay of 4 d. During intensified cleaning, the contamination level of the environment would be reduced whenever cleaning removes VRE from surfaces more rapidly than contamination occurs through diffusion. In those situations, we expect indirect transmission to be absent and indeed we observe that during the intensified cleaning period, thus indicating that colonisation is most probably due to surface contamination near the patient (Figure S1). This emphasises the importance of evacuation, cleaning, and disinfection measures that are often taken to avoid such transmission. Furthermore, a delay of 4 d implies that —in this ICU— regular cleaning of the environment (at least once a week) is sufficient to counteract diffusive delayed transmission of VRE. As noted above, indirect transmission is often caused by multiple difficult-to-quantify mechanisms. Our diffusion model provides a means to understand and quantify the expected transmission risks and the impact of control measures. Our results indicate that 2D diffusion modeling is a promising approach to describing indirect transmission in a parsimonious manner; with only a few parameters and, moreover, parameters that could feasibly be estimated. The approach was successful in explaining key features of the indirect transmission of the two bacteria studied here and can also provide an explanation of a delayed component that we identified in the transmission of VRE in an ICU.

Acknowledgments

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Appendix

Transmission experiment

Experimental design

The experiments were carried out on eight groups of broilers. Four groups were inoculated with *C. jejuni* and four groups with both *C. jejuni* and a naladixic acid resistant *E. coli*. Two of the four groups inoculated with *C. jejuni* contained five animals and two groups contained twenty animals. The same applied to the four groups inoculated with *C. jejuni* and *E. coli*. See also Table S1 for an inoculation scheme. The inoculated animals were housed together in one cage in the center of an experimental room (a separate climate controlled room in an experimental facility of the Central Veterinary Institute). Ten susceptible recipient animals were housed individually in cages surrounding this center cage placed at a distance of 75 cm (see Figure 1).

To track indirect transmission, all source and recipient animals were sampled by means of a cloacae swab (see section on Sampling). These swabs were tested for the presence of *C. jejuni* and marked *E. coli* (if applicable). Unlike *E. coli* positive animals, if a tested recipient animal was found *C. jejuni* positive, it was not only considered infected but was also immediately removed from the experiment to avoid having to deal with multiple cages contributing to the infection pressure in the analysis. The removed animals were euthanised and cecum was removed for further investigation for the presence of *C. jejuni*. The rationale not to remove *E. coli* positive animals was that it was anticipated that *E. coli* would spread faster than *C. jejuni* and removing these animals upon detection would thus interfere with the detection of onset of transmission of *C. jejuni* and the aims of the experiment. In contrast, the *C. jejuni* positive animals were immediately removed in order to keep the same design as in previous experiments with *C. jejuni* only [1].

The experiment ended 35 days post inoculation. All remaining source and recipient animals (that had not been found *C. jejuni* positive until that moment) were euthanised and cecum was removed and further investigated for the presence of *C. jejuni* and marked *E. coli* when applicable.

Animals and housing

One-day old broilers (type Ross 305) were obtained from a commercial hatchery. At day 7 and day 12 after arrival, cloacal swabs taken from each chick were used to confirm the absence of *C. jejuni* and nalidixic acid resistant *E. coli*. From the day of arrival (day 0) until 12 days post-arrival, 180 broiler chicks were housed together in one group. On day 12, the chicks were equally and randomly distributed to eight experimental rooms for the transmission experiment. Four rooms contained five source animals housed together in one center cage and ten recipient animals individually housed in ten cages surrounding the center cage as shown in Figure S1. The other four rooms contained twenty source animals housed together in one center cage and ten recipient animals individually housed in ten cages surrounding the center cage. All animals were housed on wood shavings and the drinking water was supplied through a nipple drinking system. In each set-up, the drinking nipples in the cages on the long sides of the area were supplied from one common water container while the center

cage had a separate drinking water supply. This precluded transmission via a shared drinking water system. Before the start of the experiment, all experimental rooms were cleaned and disinfected with formaldehyde. Subsequently, samples were taken from 12 different areas inside the room to check for the absence of *C. jejuni* and *E. coli*.

Inoculation

For inoculation with *C. jejuni*, the *C. jejuni* strain 356 [2] was used. The strain was freshly cultured in hearth infusion broth (microaerobically, 37°C, overnight) and diluted in buffered peptone water to obtain the intended inoculation dose (± $1 \cdot 10^6$ Colony Forming Units (CFU)/ml). The precise concentration (CFU/ml) of *C. jejuni* in the administered inoculum was determined by plating on modified cephoperazone charcoal deoxycholate agar (mCCDA) (Oxoid CM 793) with selective supplement (Oxoid CM 155) before and after the inoculation of the animals. Source animals were inoculated 14 days after arrival with 1 ml inoculum.

For inoculation with *E. coli*, a wild-type isolate was used with a point mutation in the gyrA gene, leading to a resistance to nalidixic acid (minimum inhibitory concentration > 64 mg/L). The strain was freshly cultured in normal saline solution (37°C, overnight) and diluted in buffered peptone water to obtain the intended inoculation dose (± $1 \cdot 10^6$ CFU/ml). The precise concentration (CFU/ml) of *E. coli* in the administered inoculum was determined by plating on MacConkey agar plates with 100ppm naladixic acid before and after the inoculation of the animals. Source animals were inoculated 14 days after arrival with 1 ml inoculum.

Sampling

To track indirect transmission, all animals were tested by means of a cloacae swab. After an inoculated source animal was found positive for *C. jejuni* and *E. coli* on three consecutive days, swabs for those animals were taken weekly instead of daily. For the susceptible recipient animals, swabs were taken once a day throughout the experiment. On days when both inoculated and recipient animals were to be sampled in each group, the recipient animals were sampled first. Swabs were tested within two hours after sampling in the laboratory. Samples were collected using sterile swabs (sterile plain dry swabs, Copan Diagnostics Inc., USA). For *C. jejuni* swabs were directly plated on mCCDA, incubated microaerobically at 41.5°C for 48 hours and examined for the presence of *C. jejuni*. The swab was then placed in Preston enrichment medium (Nutrient Broth no. 2, Oxoid CM0067 with *Campylobacter* selective supplement (Oxiod SR0204E) and *Campylobacter* growth supplement (Oxoid SR0232E)) and incubated microaerobically at 41.5°C for 24 hours. After incubation, it was plated on mCCDA and incubated microaerobically at 41.5°C and examined for the presence of *C. jejuni* after 24 and 48 hours. For *E. coli* swabs were directly plated on MacConkey agar with 100 ppm nalidixic acid, incubated at 37°C for 24 hours and examined for the presence of *E. coli*. The swab was then placed in a normal saline solution and incubated at 37°C for 24 hours. After incubation, it was plated on MacConkey agar plates, which were then incubated again at 37°C and examined for the presence of *E. coli* after 24 and 48 hours.

Hygienic Measures

To prevent animal caretakers from acting as a vector of transmission between experimental rooms, strict hygienic measures were used during the entire experiment. Clean coveralls were used at every entry into the experimental rooms. A pair of boots was dedicated to each room, cleaned on entering and exiting it by means of wading through a chlorinated bath (Suma Tab D4, JohnsonDiversity). To prevent direct transport from one bird to the next bird sterile gloves were changed between handling individual animals. Inoculated animals were always sampled last. Note that anyway the animal caretakers are part of the activities in the stable that can cause the diffusion within the stable.

Survival experiment

Experimental design

A separate survival experiment was carried out with four groups of five broilers each. The broilers were inoculated at age 14 days with *C. jejuni* and naladixic acid resistant *E. coli* by gavage. The groups of broilers were placed in cages in which a board (1.5 m by 1 m) was placed as a floor with normal bedding material on top. A group of broilers was put in the cage for either 24 or 72 hours. After this period the broilers were moved into another clean cage with a new board and fresh bedding material. The board floor from the emptied cage was moved from the cage including all bedding material and feces and taken into an identical experimental room with the same climate conditions as the transmission experiment described above. A wireframe grid with squares of 10 cm x 10 cm was placed over the board. Each day, starting from the day the broilers were removed from the board, a pooled sample of 10 random squares of the grid was taken. This pooled sample was immediately taken to the lab where the number of CFU's of *C. jejuni* and *E. coli* in the sample was counted (see section on Sampling for a complete description). In total 22 boards were obtained, 13 boards on which the broilers were placed for 24 hours and 9 boards with broilers placed on for 72 hours. The reasoning for 24 and 72 hours was uncertainty whether a 24 hour period would yield enough fecal material to analyse; after we finished the analysis we found no difference between samples of boards with material from 24 or 72 hour.

Animals and housing

One-day old broilers (type Ross 305) were obtained from a commercial hatchery. At day 7 and day 12 after arrival, cloacal swabs taken from each chick confirmed the absence of *C. jejuni* and nalidixic acid resistant *E. coli*. From the day of arrival (day 0) until 12 days post-arrival, 20 broiler chicks were housed together in one group. On day 12, the chicks were equally and randomly distributed into four groups of 5 animals. Each experimental room contained eight cages each measuring 1.5 by 1 meter. On the bottom of the cage, a board was placed with the same dimensions as the cage floor. Wood shavings were put on the boards as bedding material. The drinking water was supplied through a nipple drinking system. Before the start of the experiment, all experimental rooms were cleaned and disinfected with formaldehyde. Subsequently, samples were taken from 12 different areas inside the room to check for the absence of *C. jejuni* and nalidixic acid resistant *E. coli*.

Inoculation

The inoculation procedure was the same as described for the transmission experiment.

Sampling

For *C. jejuni* for the first seven days each day a sample of the boards was taken by pooling the feces from ten random 10 cm x 10 cm squares and one sample from each board on day 14. The feces inside one square were collected using tweezers to avoid too much bedding material in a sample. The pooled samples were then transported to the laboratory for further handling. In the laboratory, the samples were diluted with 500 ml buffered peptone water and the mixture was homogenised by placing them for 10 seconds in a Stomacher homogenizer (Seward Colworth Stomacher 400®). From the homogenised sample a series dilution was created by diluting 1 ml in 9 ml of normal saline solution for each step. From each dilution, 0.1 ml was plated on a mCCDA plate. The plates were then incubated microaerobically at 41.5°C for 24 hours and examined for the presence of *C. jejuni*. The number of CFU's was counted on the plate that had between 10 and 100 CFU's. The same procedure was done for nalidixic acid resistant *E. coli*, except that after day 14 every two weeks a sample was taken and each dilution was plated on MacConkey agar with 100 ppm nalidixic acid and incubated at 37°C for 24 hours after which the number of CFU's were counted.

Derivation of the diffusion model

Consider decaying particles diffusing in one dimension from a source of strength U_0 at x=0. The spatial and temporal distribution of the particles is given by Fick's second law. The partial differential equation governing the diffusion and decay process is

$$\frac{\partial u}{\partial t} = D\frac{\partial^2 u}{\partial x^2} - \alpha u, t > 0, x \in (0,\infty) \qquad \text{(S1)}$$

where D is the diffusion coefficient (m^2/day), α is the decay rate (day^{-1}), u(t,x) is the concentration at a distance x (m) from the source after time t (day). The initial and boundary conditions are $u(t,0) = U_0, \lim_{x\to\infty} u(t,x) = 0, u(0,z) = 0.$

Equation (S1) solves to

$$u(t,x) = \frac{U_0}{\sqrt{4\pi Dt}} \exp\left[-\alpha t - \frac{x^2}{4Dt}\right] \qquad \text{(S2)}$$

Equation S2 is the solution that describes the diffusive spread along the x-axis i.e., one-dimensional diffusion of a substance from a point source of an amount U_0 released at x=0 at time t=0.

For diffusion on an infinite plane surface i.e., 2D-diffusion, the concentration of the diffusing substance at a radial distance, where in this case $r^2 = x^2 + y^2$, from the source located at the point (0,0), is given by

$$S(t,r) = \frac{U_0}{4\pi Dt} \exp\left[-\alpha t - \frac{r^2}{4Dt}\right] \qquad \text{(S3)}$$

The solution $S_{cont}(t,r)$ for a continuous source emitting over a time interval $[0,\tau]$ is obtained by summing up all the contributions of the puffs emitted at the different time points taking into account the length of the diffusion period i.e., for particles emitted at $t' \in [0,\tau]$ the diffusion period is equal to $(t - t')$. The overall concentration at a radial distance r from the source is given by the convolution of the emitted quantity U_0 at time t' and the distribution $S_r(t)$ as

$$S_{cont}(t,r) = \int_0^{\tau} U_0 S_r(t-t')dt' .$$

For diffusion over a two-dimensional space from a continuous point source, the distribution of the particles is given by

$$S_{cont}(t,r) = \int_0^{\tau} \frac{U_0}{4\pi D(t-t')} \exp\left[-\alpha(t-t') - \frac{r^2}{4D(t-t')} \right] dt' \qquad \text{(S4)}$$

We note that this solution implies an infinite speed of propagation; however, this does not lead to any non-biological behavior in terms of propagation of infection. The reason for the biologically plausible behaviour being that early on (t-t' small) the transmission rates at larger distances will be negligible and thus at these distances infection events are not expected to occur. Figure S1 illustrates this: for example, at time t=1 we observe negligible infections rates for distances larger than 0.5 meter.

Replacing the continuous point source with a continuous area source, for example a rectangular cage with as coordinates for the four corners: (x_1,y_1), (x_2,y_1), (x_1,y_2) and (x_2,y_2), the concentration of particles at a given farther away location (x,y) is given by:

$$S_{cont}(x,y,t) = \int_0^{\tau}\int_{y_1}^{y_2}\int_{x_1}^{x_2} \frac{Q_0}{4\pi D(t-t')} \exp\left[-\alpha(t-t') - \frac{(x-x')^2 + (y-y')^2}{4D(t-t')} \right] dx'dy'dt' \qquad \text{(S5)}$$

where Q_0 is the source strength per unit time per unit area. This approach of extending a point source theory to an area source situation has been described before (for examples, see refs. [3-5]). If we have an area recipient, we also integrate over x and y. For example in case of a rectangular cage with (x_a,y_a), (x_a,y_b), (x_b,y_a) and (x_b,y_b) as coordinates of the four corners, we take $S_{cont}(t,r) = \int_0^{t} \frac{U_0}{4\pi D(t-t')} \exp\left[-\alpha(t-t') - \frac{r^2}{4D(t-t')} \right] dt'$.

Figure S2 shows a graph of S_{cont} in time, i.e. the amount of viable infectious material per unit area as a function of time for both *C. jejuni* and *E. coli*.

Based on the independent action hypothesis, the force of infection (FOI) experienced by a recipient animal is assumed to be proportional to the average concentration across its cage floor area, which, from Equation (S5), will tend to infinity for large t. However, even for direct transmission the rate is not infinite [6-8] therefore, it is most probably not infinite for indirect transmission. Here we hypothesise that there is a limitation on the concentration to which a recipient animal is exposed. We define that limiting value as the "exposure capacity" K of the animal. It may be governed by, among others, the mechanism of pathogen uptake as well as the accessibility of infectious material in the cage. Consequently, the FOI is taken to be proportional to the average concentration for as long as the concentration is (much) smaller

than K but for larger concentrations, it is bounded by βK. The mathematical formulation for the FOI with this behaviour is obtained from the logistic growth model theory [9] as:

$$FOI = \beta \int_{y_a}^{y_b} \int_{x_a}^{x_b} S_{cont}(x,y,t)dxdy \,/\, (1 + \int_{y_a}^{y_b} \int_{x_a}^{x_b} S_{cont}(x,y,t)dxdy \,/\, K)$$

This formulation ensures that, even in the limit of negligible pathogen decay, the infection rate will remain within biologically plausible bounds. These limitations only influence the FOI experienced by a receiving animal; it will not influence the total amount of pathogen that is accumulated at a given location at a given time. The accumulated amount is the quantity which influences the further diffusion in time and space. We assume that, for any pathogen amount, there is a non-zero probability of infection which increases exponentially fast with increasing pathogen amount. In literature, this is referred to as the dose relationship for a single-hit model [10] or the independent action hypothesis [11].

Parameter estimation

We use the Maximum Likelihood Estimation approach to estimate the diffusion coefficient D and the transmission parameters β from the data obtained in the experiments. Separate likelihood functions for *E. coli* and *C. jejuni* data were constructed because of the difference in experimental procedure i.e., chickens were removed from the experiment upon colonisation by *C. jejuni* unlike the *E. coli* colonised ones which were only removed if they also became colonised by *C. jejuni.* Using the notation

$$S'_{cont}[t, r_{i,j}, \alpha, D, K] = \int_{y_a}^{y_b} \int_{x_a}^{x_b} S_{cont}(x,y,t)dxdy \Big/ \left(1 + \int_{y_a}^{y_b} \int_{x_a}^{x_b} S_{cont}(x,y,t)dxdy \,/\, K\right)$$

in which $r_{i,j}$ denotes the distance between the cage of the source chicken j and the recipients cage i, a shorthand notation for the average across distances that is taken by the two areal integrations.

The likelihood function for the *C. jejuni* data, L_c is given by

$$L_c = \prod_{i=1}^{S_r} \left(\exp\left[-\beta_c \sum_{T=0}^{T_{\exp}} S'_{cont}[T, r_{i0}, \alpha_c, D, K] \right] \right) \prod_{j=1}^{N_d} \left(\exp\left[-\beta_c \sum_{T=0}^{dead_j} S'_{cont}[T, r_{j0}, \alpha_c, D, K] \right] \right) \times$$
$$\prod_{k=1}^{M} \left(\exp\left[-\beta_c \sum_{T=0}^{t_k - 1} S'_{cont}[T, r_{k0}, \alpha_c, D, K] \right] \times \left(1 - \exp\left[-\beta_c S'_{cont}\left[t_k, r_{k0}, \alpha_c, D, K\right] \right] \right) \right) \quad \text{(S6)}$$

The likelihood function for the *E. coli* data, L_e is given by

$$L_e = \prod_{i=1}^{M} \left(\begin{array}{l} \exp\left[-\beta_e \left(\sum_{T=0}^{t_i - 1} S'_{cont}\left[T, r_{i0}, \alpha_e, D, K\right] + \sum_{j=1}^{i-1} \sum_{T=t_j}^{\min[t_i - 1, culled_j]} S'_{cont}\left[t_i - T, r_{ij}, \alpha_e, D, K\right] \right) \right] \times \\ \left(1 - \exp\left[-\beta_e \left(S'_{cont}\left[t_i, r_{i0}, \alpha_e, D, K\right] + \sum_{j=1}^{i-1} S'_{cont}\left[t_i - t_j, r_{ij}, \alpha_e, D, K\right] \right) \right] \right) \end{array} \right) \quad \text{(S7)}$$

S_t is the total number of susceptible chickens that escaped from infection throughout the experiment. In the *E. coli* data there are no animals escaping from infection throughout the

experiment (S_t=0), which is why the first factor in Equation S6 has no counterpart in Equation S7. T_{exp} is the number of days in the experiment. N_d is the total number of animals that died due to other causes than removal (during the complete experiment 9 animals died to other causes than removal). $dead_j$ is the day that animal *j* died due to other causes. *M* is the total number of transmission events that occurred, t_i is the day that the *i*-th transmission event occurred $culled_j$ is the day that chicken *j* was culled.

The factors in L_c (Equation S6) are described as follows;

$$\prod_{i=1}^{S_t}\left(\exp\left[-\beta_c\sum_{T=0}^{T_{exp}}S'_{cont}\left[T,r_{i0},\alpha_c,D,K\right]\right]\right)$$

is the probability of escaping infection throughout the experiment for all escapees,

$$\prod_{j=1}^{N_d}\left(\exp\left[-\beta_c\sum_{T=0}^{dead_j}S'_{cont}\left[T,r_{j0},\alpha_c,D,K\right]\right]\right)$$

is the probability of escaping until the animal died due to other causes,

$$\prod_{k=1}^{M}\left(\exp\left[-\beta_c\sum_{T=0}^{t_k-1}S'_{cont}\left[T,r_{k0},\alpha_c,D,K\right]\right]\times\left(1-\exp\left[-\beta_c S'_{cont}[t_k,r_{k0},\alpha_c,d,K]\right]\right)\right)$$

is the probability of getting infected on day *t* after escaping *t-1* days. For the factors in L_e (Equation S7), is the probability of escaping infection from the inoculated and the contact-infected animals and

$$\left(1-\exp\left[-\beta_e\left(S'_{cont}\left[t_i,r_{i0},\alpha_e,D,K\right]+\sum_{j=1}^{i-1}S'_{cont}\left[t_i-t_j,r_j,\alpha_e,D,K\right]\right)\right]\right)$$

is the probability of being infected by either the inoculated or contact-infected animals. The estimates for the parameters *D*, β_c and β_e are those that maximize the likelihood of observing the data from the experiments given the functions L_c and L_e. We obtain the 95% confidence intervals for the maximum likelihood estimates *D*, β_c and β_e using the likelihood ratio test; for each parameter univariate confidence bounds were calculated.

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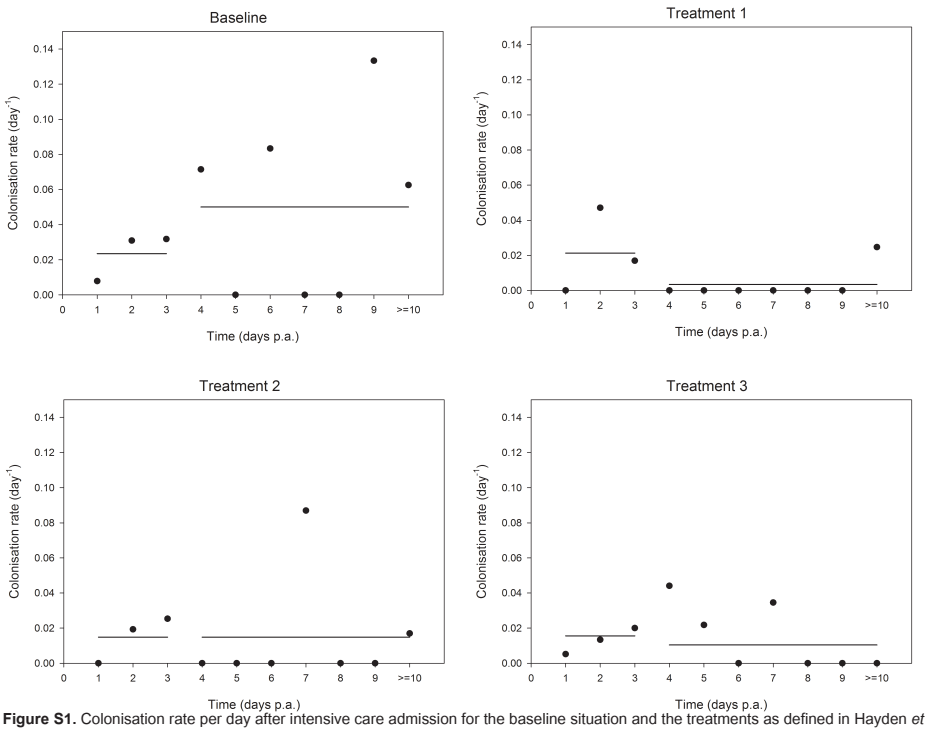

Figure S1. Colonisation rate per day after intensive care admission for the baseline situation and the treatments as defined in Hayden *et al* [12]. Day 0 is the day of admission. Solid lines indicate average colonisation rate for that period. p.a. = post admission.

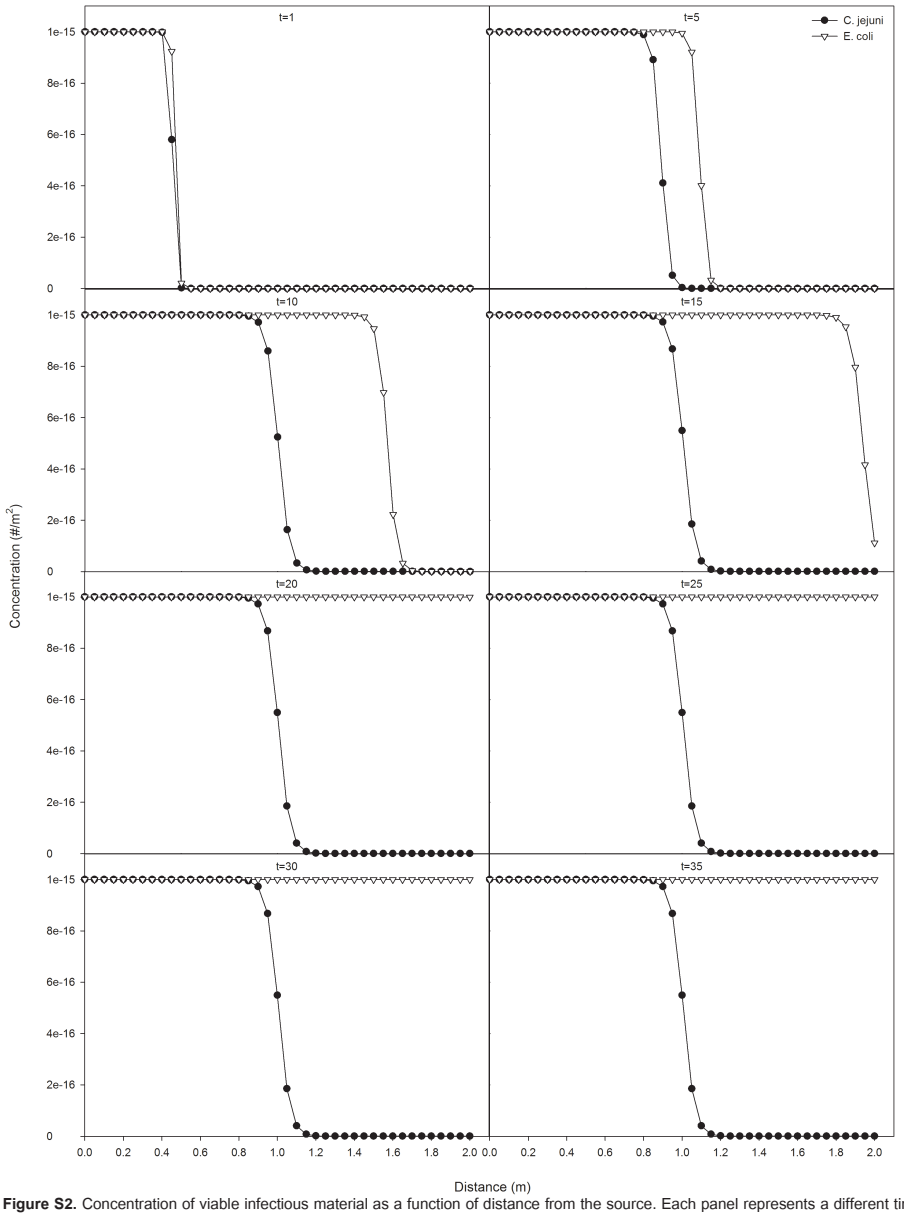

Figure S2. Concentration of viable infectious material as a function of distance from the source. Each panel represents a different time of observation. Parameter values used: D=0.0025 m^2/day, α_{campy}=2.25 day^{-1}, α_{coli}=0 day^{-1},K=1·10^{-15}.

Table S1. Inoculation scheme of the indirect transmission experiment

Group	Inoculum	Animals inoculated
1	*C. jejuni*	5
2	*C. jejuni* & *E. coli*	5
3	*C. jejuni*	5
4	*C. jejuni* & *E. coli*	5
5	*C. jejuni*	20
6	*C. jejuni* & *E. coli*	20
7	*C. jejuni*	20
8	*C. jejuni* & *E. coli*	20

Table S2. Summarised results from the survival experiment

Day	C. jejuni (CFU)	E. coli (CFU)
1	$1.96 \cdot 10^{6}$	$1.29 \cdot 10^{8}$
2	$2.04 \cdot 10^{5}$	$1.37 \cdot 10^{8}$
3	$2.74 \cdot 10^{4}$	$1.66 \cdot 10^{8}$
4	$2.27 \cdot 10^{2}$	$1.76 \cdot 10^{8}$
5	$1.09 \cdot 10^{2}$	$8.23 \cdot 10^{7}$
6	0	$1.32 \cdot 10^{8}$
7	0	$1.02 \cdot 10^{8}$
14	0	$5.28 \cdot 10^{7}$
21	0	$9.70 \cdot 10^{6}$
28	0	$7.66 \cdot 10^{7}$
35	0	$1.01 \cdot 10^{8}$
>35	0	$4.97 \cdot 10^{7}$

Per bacterium species (*C. jejuni* and *E. coli*) counts of the average number of colony forming units (CFU) for each day are reported.

CHAPTER 6

Mathematical models for the response of broilers to different doses of *Campylobacter jejuni*

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Abstract

An important factor in the transmission of an infectious disease is the probability of a pathogen to infect a host. This probability is generally estimated from dose-response experiments. To assess the response of broilers to different doses of *Campylobacter jejuni* (*C. jejuni*) a dose-response experiment was carried out in this study. Furthermore the effect of acidification of drinking water on the response of broilers to inoculation with different doses of *C. jejuni* was assessed as a possible control measure. In this study different dose-response models were constructed and the fit of these models to the obtained experimental data was tested using a maximum likelihood approach. The results show that a model that satisfies the independent action hypothesis but includes heterogeneity results in a better fitting model for the data acquired. Furthermore, it was shown that acidification of the drinking water has no significant effect on the response of broilers to inoculation with different doses of *C. jejuni*.

Introduction

Dose-response experiments measure how the probability of a certain response (death, disease, sign of infection, immune response), observed when an animal is inoculated once via one particular route with a certain amount (called dose) of an infectious agent, depends on that dose. With these studies we can estimate the relative infectiousness of different pathogen doses [1-7].

For such a dose-response relationship it is typically assumed that the proportion of hosts that respond to (get infected by) exposure to an infectious agent increases with an increasing dose. Each pathogen-host combination may have a unique dose-response relation. This relation is based on the characteristics of both the pathogen, the host and the inoculation route. Characteristics of the pathogen include infectiousness of the pathogen to the specific host and the method of reproduction within the host, while the characteristics of the host include factors such as susceptibility to the pathogen and host responses, for example from the immune system.

Most recent theoretical models are based on the assumptions that each infectious particle has a non-zero probability of infecting a host and that the probability of infection increases with the number of particles [8]. In the recent past, a number of dose-response models have been used, ranging from minimum infectious dose concepts to the single hit models based on the independent action hypothesis (IAH) [8-11]. Mathematical models provide an explanation for the characteristics of the infectious agent and the shape of the observed dose-response curve and especially in situations where the inoculation dose or exposure dose is low, it is important to have a (theoretical) model that gives a biologically correct relation between dose and response so that also extrapolations are correct. This because in those situation where the dose is very low, the probability of infecting a host is also very low and to accurately estimate these probabilities too many trials are needed. Therefore the probabilities for low doses are usually extrapolated from intermediate doses, as for these doses there are more (informative) observations.

In this study we will use colonisation of broilers by *Campylobacter jejuni* (*C. jejuni*) as a study system and explore the fit of different mathematical dose-response models to data obtained from a series of dose-response experiments. In these experiments groups of broilers were inoculated with different doses of *C. jejuni* and the response was measured by taking daily cloaca swabs of all broilers and analysing the results of the swabs in the laboratory. Furthermore the effect of acidifation of drinking water on the response of broilers to inoculation with different doses of *C. jejuni* was assessed as a possible control measure. In broiler chickens the use of fermented liquid feed has been shown to reduce the susceptibility to *C. jejuni* and *Salmonella* [12-14]. Previous research showed that the effects of fermented liquid feed are attributed to the high level of organic acids and the low pH of this feed. Following this line of reasoning, acidified drinking water may be expected to have a similar effect on the susceptibility of broilers to *C. jejuni* as fermented liquid feed has. It is thus possible that acidification of the drinking water can be used as a modulation factor for transmission. In this paper we will, in a systematic fashion, construct different dose-response models and test the fit of these models to the obtained experimental data using a maximum likelihood

approach. We show that a model that satisfies the independent action hypothesis but includes heterogeneity results in a better fitting model for the data acquired and we will discuss the biological implications of this result. Furthermore we show that acidification of the drinking water has no significant effect on the response of broilers to inoculation with different doses of *C. jejuni*.

Materials and methods

Dose-response experiments

Study design

For this study a series of dose-response experiments were carried out. Each experiment consisted of 1 control group and 4 dose groups. The treatment groups received different inoculation doses of *C. jejuni*. The (intended) administered doses were: 10^1; 10^2; 10^3 and 10^4 colony forming units (CFU's) of *C. jejuni* per ml. Each dose group consisted of 9 animals and the control groups consisted of 4 animals, resulting in a total of 36 animals per dose group and 16 animals in the control group. All animals arrived at the experiment facility as one-day old animals. After 7 days the animals were uniquely tagged with a wing tag. After a 14 days rearing period in which the animals were housed together the animals were relocated to the experimental room. Each animal was inoculated with the intended dose and placed in its own cage. The animals of the different doses and the control group were randomly distributed over all available cages. The housing consisted of so-called battery-cages.

From the day of inoculation, each day a cloaca swap was taken from each animal. These swabs were then transported to the laboratory within two hours where the swabs were analysed for the presence of *C. jejuni* (see section on Sampling for complete procedure). Cloaca samples were taken for a period of seven days, after which the animals where euthanised and send to the laboratory for further analyses.

Acidification of the drinking water

A second experiment using the exact same setup was carried out simultaneously with the only difference being the drinking water the animals received. In this second experiment the animals received acidified drinking water instead of tapwater. A commercially available acid (Forticoat®, Selko BV) was diluted until a final pH of 4 (approximately 2 ml acid on 1 l water). Active ingredients of the commercially available acid are: sorbic acid, formic acid, acetic acid, lactic acid, propionic acid, ammonium formate, L-ascorbic acid, citric acid, mono- and diglycerides of edible fatty acids and 1,2-propanediol.

Animals & Housing

One-day old broilers (type Ross 308) were obtained from a commercial hatchery. All animals were uniquely tagged so they could be tracked throughout the experiment. At day 7 and day 12 after arrival, cloacal swabs taken from each animal confirmed the absence of *C. jejuni*. For each of the four experiments from the day of arrival (day 0) until 12 days post-arrival, 40 animals were housed together in one experimental room. On day 14 each animal was inoculated by gavage with the intended dose and placed

in an individual cage. All animals were housed on a rubber matting to avoid feet problems. Drinking water and food was supplied *ad lib*. Drinking water was administered via drinking nipples which were supplied via one common water container. Before the start of the experiment, all experimental rooms were cleaned and disinfected with formaldehyde. Subsequently, samples were taken from 12 different areas inside the room to check for the absence of *C. jejuni*.

Inoculation

Broilers, aged 14 days, were orally inoculated by gavage, while fixating the animal, to bring 1 ml of buffered peptone water containing the intended inoculation dose in the bird's crop. For inoculation, the *C. jejuni* strain 356 was used [15]. The strain was freshly cultured in hearth infusion broth (microaerobically, 37°C, overnight) and diluted in buffered peptone water to obtain the intended inoculation dose. The precise concentration (CFU/ml) of *C. jejuni* in the administered inoculum was determined by plating on modified cephoperazone charcoal deoxycholate agar (mCCDA) (Oxoid CM 793) with selective supplement (Oxoid CM 155) before and after the inoculation of the animals.

Sampling

Samples were collected using sterile swabs. Swabs were directly plated on mCCDA, these plates where incubated microaerobically at 41.5°C for 48h and examined for the presence of *C. jejuni*. After plating the swabs were placed in an enrichment medium (CCD broth) and incubated microaerobically at 41.5°C for 24h. After incubation 10 µl was plated on mCCDA and incubated microaerobically at 41.5°C for 48h and examined for the presence of *C. jejuni*. Sensitivity and specificity for testing cloaca swabs were estimated as both being very close to 1 (personal communication R. van der Hulst, CVI, Lelystad).

Ethics Statement

Animal experiments within the Animal Science Group (ASG) of Wageningen UR are performed in accordance with the provisions of the European Convention for the protection of vertebrate animals used for experimental and other scientific purposes (86/609 EG). In accordance with the Act on Experimental Animals of the Netherlands the use of experimental animals is granted to ASG by permit from the Dutch Government (licence DLO dd. 17 Feb. 2010. Licence nr. 40100). The protocol was approved by the Committee on the Ethics of Animal Experiments of the ASG.

Dose-response models

In this study we will formulate a number of different dose-response models that have distinct underlying biological mechanisms and fit these models to the data of the experiments. We will construct the different candidate models here and explain the assumptions made and the conditions needed for all candidate models.

Exponential model

The Independent Action Hypothesis (IAH, or single hit model) states that each infectious particle in an inoculum acts independently and has a non-zero probability of causing an infection [8]. When we assume that each individual particle has the same probability of infection, independent of the individual host, the IAH leads to the exponential dose-response relationship:

$$P_{\text{inf}}^{\text{exp}}(D,r) = 1 - e^{-r \cdot D} \quad (1)$$

where r is the probability of infection. To keep the parameters of the model comparable to the other models used in this study we have defined r as $ln(2)/D_{50}$, resulting in:

$$P_{\text{inf}}^{\text{exp}}(D,D_{50}) = 1 - e^{-ln(2)\frac{D}{D_{50}}}$$

Here D_{50} is that dose where 50% of the challenged animals become colonised.

Hypergeometric model

It is, however, possible that there is variation in r, due to variation between pathogen particles and/or between hosts. This variation can be described by a probability distribution with density function $f(r,\theta)$.

Including this variation in Equation 1, the probability of infection then becomes:

$$P_{\text{inf}}(D,\theta) = \int_{r=0}^{1} 1 - e^{-r \cdot D} f(r,\theta) dr \quad (2)$$

If we choose a Beta-distribution for the variation, the solution of Equation 2 is:

$$P_{\text{inf}}^{\text{hyp}}(D,\alpha,\beta) = 1 - {}_1F_1(\alpha,\alpha+\beta,-D) \quad (3)$$

where ${}_1F_1()$ is the Kummer confluent hypergeometric function.

Weibull model

Here we extended the exponential dose-response model (Equation 1) by including the parameter α as a shape parameter (α > 0), resulting in:

$$P_{\text{inf}}^{\text{wei}}(D,D_{50}) = 1 - e^{-ln(2)\left(\frac{D}{D_{50}}\right)^{\alpha}}$$

This form is also known as the Weibull dose-response model, an empirical model that does not satisfy the IAH (unless α = 1). We included a factor $ln(2)$ in the model for D_{50} to have the same meaning as in the Sigmoid model.

Sigmoid model

Lastly, another possible empirical model (that does not satisfy the IAH) for a dose-response equation is a sigmoid function:

$$P_{inf}^{sig}(D,D_{50}) = 1 - \frac{1}{1+\left(D/D_{50}\right)^{\alpha}}$$

Here is the shape parameter (α > 0). That this model does not satisfy the IAH can be seen for example by noting that for α > 1 and for low doses, a doubling of the dose (for small doses) results in a more than doubled probability of infection, which is only possible if we assume there is some form of synergy (or interaction) between particles.

Model fitting

The probability of observing *k* successful inoculations (*I*) with dose *D*, given a dose-response function $P_{inf}(D,\alpha,\beta,...)$ takes the form:

$$P(I=k)=\binom{N}{k}\cdot P_{inf}\left(D,\alpha,\beta,...\right)^{k}\cdot\left(1-P_{inf}\left(D,\alpha,\beta,...\right)\right)^{N-k}$$

For multiple dose groups we thus get:

$$\prod_{i=1}^{N_d} P\left(I=k\mid D_i\right) \qquad (4)$$

where N_d is the total number of inoculations per dose.

Using the data of the animal experiments, the different parameters of the dose-response functions can be estimated by maximising Equation 4. We obtain the 95% confidence intervals around the maximum likelihood estimates using the likelihood ratio test; for each parameter univariate confidence bounds were calculated.

As a measure for the goodness of fit of the models we used Akaike's Information Criterion (AIC) [16]. The AIC is calculated as $2k-2\log(L_{max})$, where *k* is the number of estimated parameters in the model and L_{max} is the maximised value of the likelihood function for the model. The lowest AIC score indicates the (mathematically) preferred model. To compare different plausible models to each other we considered the ΔAIC score calculated as the difference with the model with the lowest AIC. We used the criterion by Burnham and Anderson [16] for further selection between models: i.e. only ΔAIC > 2.0 indicate a possible difference in model fit.

Results

Animal experiment

The results from the dose-response experiments are given in Table 1. The results show that the number of animals that are infected in a seven-day period is increasing with increasing dose, as expected. Furthermore we observed a small difference between the number of infected in the tapwater group compared to the acidified drinking water group; in the group that received acidified drinking water less animals were infected in all dose groups.

Table 1: Number of animals infected per dose per treatment.

Dose (CFU's/ml)	Total animals (per treatment)	Animals infected	
		Tapwater	Acidified water
0	16	0	0
10^1	36	3	1
10^2	36	3	0
10^3	36	9	8
10^4	36	22	18

Model fitting results

All models described in the methods section were fitted to the data obtained from the animal experiments and the goodness of fit of each model was compared using the AIC as described in the methods section. The results of this fitting are described in Table 2 and Figure 1. From Table 2 it is clear that the Sigmoid and the Weibull model give the best (mathematical) fit to the data. These models are therefore the preferred models for interpolation between the doses used in this study. If however we wish to extrapolate to lower dosages a biologically correct model would be necessary. The models that do adhere to the IAH (both the Exponential model and the Hypergeometric model) would thus be needed to extrapolate to higher or lower doses. As ΔAIC differs less than 7 for the Hypergeometric model it implies that this model also gives a reasonable model fit. Also from Figure 1 we can see that the best fitting models are the Sigmoid model and the Weibull model.

Effects of acidification of the drinking water

As to the effects of acidification of the drinking water on the response to inoculation dose, based on the odds ratio of 0.59, 95% C.I.:[0.31 - 1.12], a protective effect of acidification of the drinking water can be observed. However due to the relatively small dataset this protective effect is not statistically significant (p = 0.11).

Discussion

Here we studied the dose-response of broilers to different doses of *C. jejuni* using dose-response experiments. To this end we considered different models to describe this dose-response relationship. In these experiments the effect of acidification of the drinking water on the response inoculation with different doses of *C. jejuni* was also assessed. The relative goodness of fit of the models was tested by fitting them to experimental data on the response of broilers to different doses of *C. jejuni*. To this end data from the dose-response experiments was analysed using a maximum likelihood approach. Regarding the different hypothetical models we found that the models that fitted best to the data were the Weibull model (AIC=135.57) and the Sigmoid model (AIC=136.28). No further preference for one of the models could be inferred from the differences in AIC scores. The mathematical fit of these two models is thus similar. Examination of the equations for the two best fitting models reveals

Table 2. Results of the model fitting

		Tap water			Acidified water		
Model	**Parameter**	**Estimate**	**C.I.**	**AIC**	**Estimate**	**C.I.**	**AIC**
Exp	D_{50}	4869.5	[3524 - 6942]	163.95	7502.1	[5208 - 11307]	112.19
IAH	α	0.137	[0.062 - 0.344]	140.92	0.252	[0.096 - 0.982]	106.08
	β	47.43	[3.12 - 486.44]		663.00	[65.09 - 6737.36]	
Weibull	D_{50}	5670.84	[2690 - 19153]	135.57	9326.94	[4971 - 27679]	103.84
	α	0.423	[0.267 - 0.624]		0.581	[0.367 - 0.848]	
Sigmoid	D_{50}	5771.65	[2350 - 25209]	136.28	9374.55	[4391 - 33496]	103.68
	α	0.490	[0.304 - 0.704]		0.668	[0.416 - 0.980]	

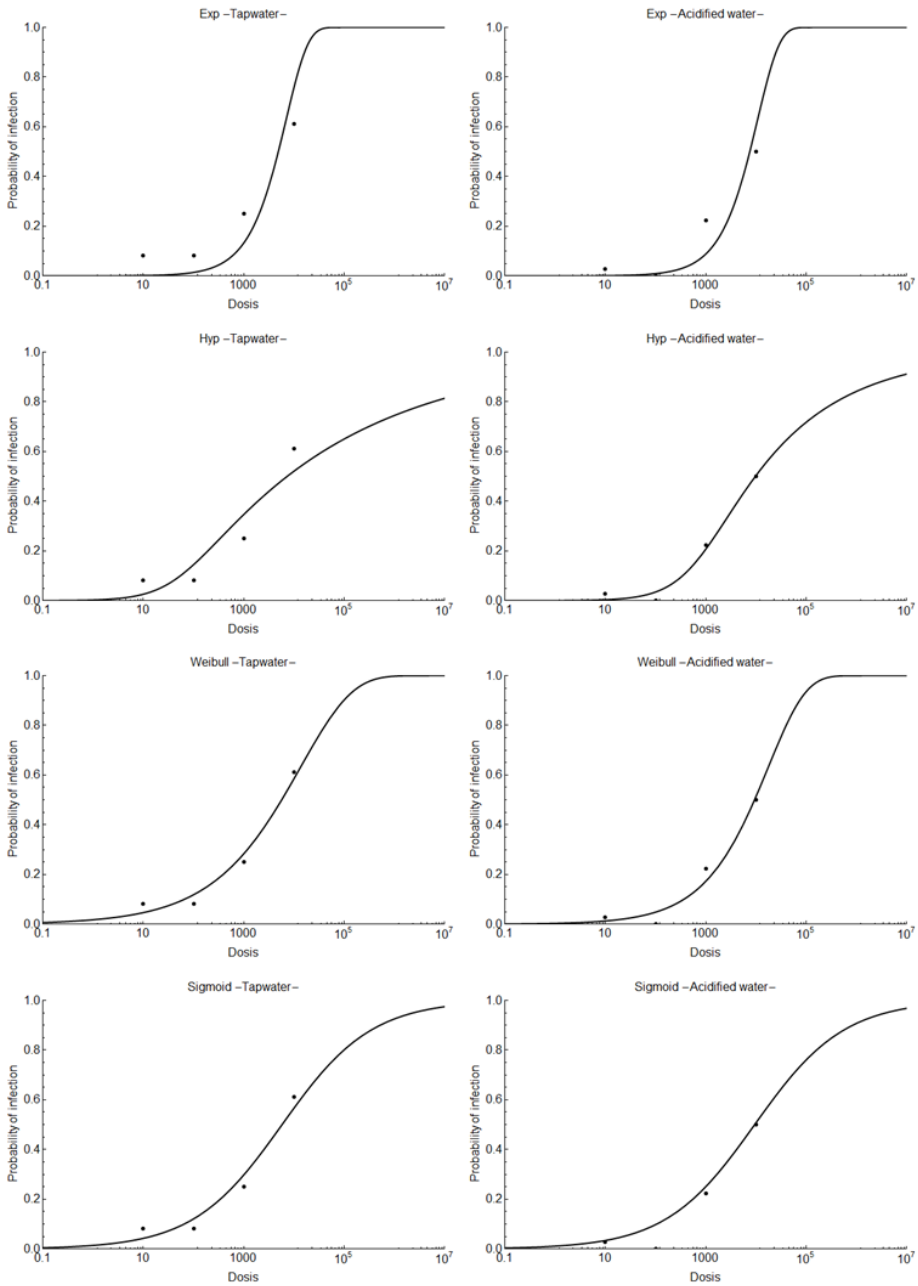

Figure 1. Resulting fitted dose response curves

that the two models are quite similar, both have a form of $1-\frac{1}{x}$, where *x* is either $1+(\frac{D}{D_{50}})$ for the Sigmoid model or $e^{\frac{D}{D_{50}}}$ for the Weibull model. Therefore we do not expect the AIC scores to be much different.

What is more striking is that the two models we propose here have better (lower) AIC scores than the IAH models. Although there is no clear biological reason for this better fit it is most probably due to the fact that the two proposed models are more flexible, despite having the same number of parameters. If, however, we want the extrapolate to lower doses (common in, for example, indirect transmission) a biologically correct model is needed. The only two models considered here that do adhere to the IAH are the Exponential model and the Hypergeometric model. And for extrapolation one of these two models should thus be used. Comparing the AIC of those two models shows that the Hypergeometric is the better fitting one (AIC=140.92 vs. 163.95). We thus conclude that although mathematically the Sigmoid and Weibull model fit better, the Hypergeometric model should be chosen for extrapolation to doses that have not been measured.

The absence of significant effect of acidification of the drinking water is in correspondence with previous findings. For direct transmission we already found that acidification of the drinking water has no effect on the rate of transmission compared to a group receiving normal tapwater [17]. For indirect transmission however, it was found that acidification of the drinking water lowers the transmission rate. Our results indicate that direct administering of doses as low as 10^1 CFU's per ml are similar to direct transmission. Possibly the doses that are associated with indirect transmission are even lower and therefore the acidification of the drinking water has no significant measurable effect in our dose-response experiment. Low doses are not easy to measure in an experimental setting. The probabilities associated with these low doses thus need to be extrapolated from measurements from higher doses. Therefore the dose-response model that is used to this end should not only fit well but it should also be biologically correct as in this situation the probability of infection is easily over- or underestimated. Another explanation can be that the time period over which the dose is ingested in an indirect transmission setting is different, i.e. it could be that during a whole day an animal does take up 10^1 CFU's however this could be distributed over the whole day as 10 independent intakes of 1 CFU. A possible explanation for this is that there is clustering of pathogen in the administered dose, for example due to bio-film formation. The outer pathogens of a cluster could act as a buffer (or protective layer) for the inner cells against "threats" from the outside, such as the acids in the stomach fluid, etc. Possible models to use for this situation include multi-hit models [18] and dose-time-response model [19]. It should be noted that these models do not abide the IAH. Yet another possibility is that the effect of acidification is not sorted at the receiving side of the transmission, but only at the sender side and therefore we do not see an effect in our study. However, this is contradicted by a previous study, where we found no difference in the effect between acidification at the sender and at the receiver side [20].

In conclusion we have identified two candidate models that accurately describe the relationship

between administering a certain dose of *C. jejuni* and the response of broilers to that dose. If used for interpolation these models are the best options of the models considered here. However if extrapolation to doses outside the measured range is needed, the best model is the Hypergeometric model because this model does abide the IAH and is thus biologically correct (under the assumption that the IAH is correct). Furthermore no statistically significant effect of acidification of the drinking water was found, although the odds ratio does show that there is a trend towards a protective effect of acidification of the drinking water.

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CHAPTER 7

General Discussion

Introduction

Transmission between spatially separated hosts is a common problem, not only during major outbreaks of livestock diseases, but also in many other settings such as the transmission of infectious diseases between plants and crops or in healthcare settings [1-7].

The central motivation for the research described in this thesis were the disease transmission events that occurred during the last major epidemics of livestock diseases (e.g. Foot and Mouth Disease Virus (FMDV) and Classical Swine Fever Virus (CSFV)) in The Netherlands and abroad, despite movement bans and other (bio-)security measures. The occurrence of transmission despite the existing movement bans implies that there was unreported illegal transport (which is not very likely) and/or that the pathogen is spread by indirect transmission (transmission with no direct host-host contact). This last form of transmission is often termed "neighbourhood transmission" [8-12]. Between-farm transmission often consists of a combination of direct and indirect transmission, direct transmission in the form of animal movements between farms, and indirect transmission, via for example contaminated shared personnel or equipment, or other fomites. During an epidemic however there is a standstill of all movements between farms, minimising the risk of infection through direct transmission. Although (individual) routes of indirect transmission have been studied in more detail [11, 13, 14] the underlying mechanisms are not well understood.

The ultimate goal is to improve current bio-security-based intervention measures against the transmission of livestock diseases which also imply better prevention of indirect transmission. However, only little is known about indirect transmission of diseases between animal hosts. Therefore, as a first step, better understanding of the processes and mechanisms that underlie this form of transmission and quantitative estimates of (indirect) transmission parameters are needed.

To study indirect transmission in more detail it is convenient to think of this process as three distinct sub-processes (Figure 1):

1. an infectious "sender" excretes the pathogen; after which
2. the pathogen is transported via some route through the environment to a susceptible "receiver"; and subsequently
3. the receiver becomes infected by the pathogen.

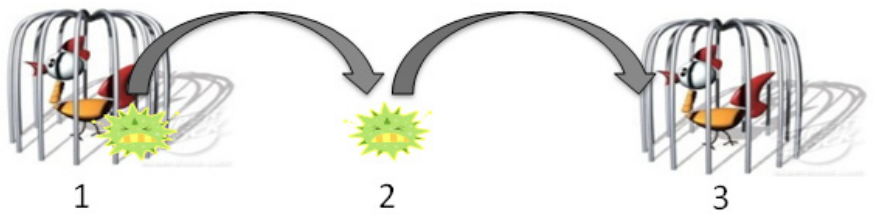

Figure 1. Schematic overview of the sub-processes of indirect transmission. (1) an infectious "sender" excretes the pathogen, after which (2) the pathogen is transported via some route through the environment to a susceptible "receiver", and subsequently (3) the receiver becomes infected by the pathogen.

To gain more insight in the underlying mechanisms of transmission between spatially separated hosts a combination of novel transmission experiments and tailor-made mathematical models were used in this thesis.

Summary of findings

The goal of this thesis was to gain more insight in the underlying mechanisms of transmission between spatially separated hosts (i.e. indirect transmission). As discussed above, this form of transmission frequently occurs in outbreaks of major livestock diseases in the form of neighbourhood transmission. To study the mechanisms of indirect transmission in more detail a factor to modulate transmission was used that could potentially help to gain insight in the separate sub-processes of indirect transmission. In broiler chickens the use of fermented liquid feed has been shown to reduce the susceptibility to *Campylobacter jejuni* (*C. jejuni*) and *Salmonella* [15-17]. Previous research showed that the effects of fermented liquid feed are attributed to the high level of organic acids and the low pH of this feed. Following this line of reasoning, acidified drinking water may be expected to have a similar effect on the susceptibility of broilers to *C. jejuni* as fermented liquid feed has. It is thus possible that acidification of the drinking water could be used as a modulation factor for transmission. Therefore the effect of acidification of the drinking water on the transmission parameters of direct and indirect transmission was studied in chapter 2. The results of this study show that acidified drinking water has an effect on indirect transmission but not on direct transmission of *C. jejuni* between broilers. Furthermore, it shows that, in terms of the transmission parameter β, there is a difference between direct and indirect transmission, with the estimated value for β for indirect transmission being two orders of magnitude lower than for direct transmission (β = 3.7 day^{-1}; 95% confidence interval (C.I.): [2.0 - 6.8] for direct transmission versus β = 0.011 day^{-1}; 95 C.I.: [0.0006 - 0.047] for indirect transmission). Thus, indirect transmission occurs at a lower rate than direct transmission.

The sender and receiver sub-processes (sub-process 1 and 3 in Figure 1) are studied in more detail in chapter 3; showing that a significant negative interaction effect between acidification of the sender and receiver sub-processes exists. This indicates that there is no additional effect of acidification of the drinking water on both sides of the transmission process compared to acidified drinking water only on one side.

To study the transport of the pathogen in the environment (sub-process 2 in Figure 1) in more detail, a series of indirect transmission experiments was carried out. Furthermore a model framework was developed to study indirect transmission between spatially separated hosts. This model framework is setup as a compartmental model in which the time the pathogen has spent in the environment outside the host is explicitly taken into account by one or more separate environmental reservoirs. The data from the transmission experiments that were conducted show a large delay before the first transmission event and a subsequent clustering of transmission events that take place after the first event. This transmission pattern is best described by a multistage environmental route from sending to receiving animal, which suggests that indirect transmission occurs through progressive (but slow) contamination of the environment surrounding the source (chapter 4). The best fitting, resulting model

also identifies the decay rate of a pathogen in the environment outside the host as a key parameter. Based on this, one of the predictions of this model is that the observed delay in onset of transmission is dependent of the decay rate of the pathogen. To formulate this more precisely: the model predicts that the faster a pathogen decays in the environment the longer the delay before onset of first transmission will be.

To test the prediction that the delay before onset of transmission is dependent on the decay rate of the pathogen the indirect transmission experiments where repeated with both *C. jejuni* and (a marked) *Escherichia coli* (*E. coli*) in chapter 5. In this experiment broiler chickens where inoculated with only *C. jejuni* or with both *C. jejuni* and *E. coli*. The results of this experiment showed that for *C. jejuni* it takes much longer to cross the small distance of approximately 75 cm for the first time than it does for *E. coli*. As both pathogens are excreted in similar amounts and are transported on the same material (feaces) any difference in delay before onset of transmission are attributable to the difference in decay rate of the two pathogens in the environment. A general model was developed, capable of explaining the observed patterns from both pathogens. Not only does this model explicitly take into account the distance between hosts; it also takes into account the amount of time a pathogen spends (travels) in the environment. During this travel time the pathogen is decaying. The resulting model describes the spread of infectious material in the environment with the aid of a two-dimensional (2D) diffusion model. Predictions of this model were also tested using data of Vancomycin-Resistant *Enterococci* in a hospital intensive care unit. Again the model predicts a delayed transmission component and indeed the existence of this component is shown in such a setting. When a thorough cleaning regime is applied, this delayed component is not observed anymore and thus the transmission risk is reduced.

The above model explains the delayed transmission from a delayed increase in pathogen concentration at the distant location/cage (Figure 3 in chapter 5). Based on a plausible dose-response assumption the model predicts that this delayed increase in concentration leads to a period with “zero” infection rate followed by a sharp onset of a period with non-zero rate of infection.

The dose-response assumption of the model of chapter 5 is that the infection hazard of the recipient host is proportional to the pathogen concentration in its location/cage. It can be shown that this assumption, if one also assumes that the rate at which pathogens are entering the host is proportional to the local concentration in the environment, corresponds to assuming the exponential dose-response relationship. This exponential relationship is equivalent to assuming that (1) each single bacterium entering the host represents an independent probability of infection of the host, and (2) this probability is the same for each individual bacterium. In chapter 6 the form of the dose-response curve is studied in detail for *C. jejuni* by carrying out a dose-response experiment. In this chapter a range of dose-response models were compared and tested how well these fitted to the data from the dose-response experiment. Here it was shown that for interpolating purposes two relatively simple models are best capable of describing the data from the dose-response experiment. However these models do not abide the independent action hypothesis that each pathogen particle acts independently of other particles. For extrapolating purposes, however, it was shown that

from the models that were studied a hypergeometric dose-response model is the best fitting model. This model does abide the independent action hypothesis and is thus assumed to be more biologically correct for low doses.

Modelling transmission between spatially separated hosts

Most infectious diseases are treated as being directly transmitted in existing transmission models. The assumption for this is that if an environmental stage exists between a sender and a receiver animal and it is short enough (both in distance and time) that this can be safely ignored. It is thus assumed that there is an almost instantaneous transmission of the pathogen between hosts and a standard SIR-framework [18-20] can be used to model disease spread and to estimate transmission parameters.

In this thesis, however, transmission between spatially separated hosts is studied. This means that there is a physical barrier between sender and receiver animals and no direct contact is possible. The results from the transmission experiments conducted in chapters 4 and 5 show a significant delay before onset of transmission, followed by subsequent clustering of transmission events. This implies that this pattern cannot be described by instantaneous transmission with a lower value for the transmission value β. With a lower value for β the delay before first onset of transmission would have been the same as the average time between every other two infection events i.e. between the first and second infection event etc. This is not the case as the events are more clustered than that.

This also means that the standard SIR-framework cannot be used. Therefore a different class of models was developed in chapter 4 and further extended in chapter 5. The models developed in these chapters are quite similar; they both assume that infectious material is present in the environment before transmission takes place. Furthermore they both assume that there is decay of the pathogen during the time spend in the environment. The model in chapter 5 however explicitly takes distance into account, something that is not incorporated explicitly in the models in chapter 4. The best-fit model in chapter 4 can be seen as a discretised form of the model in chapter 5.

Although it is assumed that infectious material is present in the environment before transmission takes place, it takes infectious material four days to cross the distance of approximately 75 cm and to build up levels high enough to cause infection. This is the case for *E. coli*. As is shown in a separate experiment in chapter 5, *E. coli* can survive for more than 100 days in the environment outside the host and thus for the duration of the experiment the decay rate is negligible. For *C. jejuni* the decay rate is not negligible and this causes the delay before onset of transmission for *C. jejuni* to be much larger: it takes thirteen days for *C. jejuni* to reach levels high enough to cause infection at 75 cm. This model thus makes it possible to accurately describe the delay and clustering observed in the transmission experiments for different pathogens, using readily estimable parameters.

Controlling indirect transmission

Acidification of drinking water and feed as a measure to decrease the susceptibility of broilers to *C. jejuni* and *Salmonella* has been studied before in different experimental settings, with

promising results [15, 21-25]. As shown in chapter 2, acidification of the drinking water decreases indirect but not direct transmission of *C. jejuni* between broilers. This difference in effect between direct and indirect transmission is most likely to be the result of different levels of exposure of the hosts to the pathogen; with indirect transmission the exposure dose is expected to be much lower compared to direct transmission. Although great care should be taken with extrapolating the transmission in the experimental setup to between-farm transmission, the results imply that it might be possible to delay the introduction of *C. jejuni* in a fully susceptible flock by acidifying the drinking water of broilers. However, once it is introduced in a flock, *C. jejuni* will spread very fast by a combination of direct and indirect transmission. To evaluate the effects of acidification of the drinking water, a similar method as in chapter 5 for the intensive care unit data can be used.

When studied in more detail in chapter 3 acidification of the drinking water has an equal effect on both sender and receiver animal, but acidification of both the sender and receiver does not have an added effect (or more accurately: there is a significant negative interaction effect). It is hypothesised in chapter 3 that this negative interaction effect is caused by selection pressure of the pathogen on the sender side. This indicates that it may be too simple to model indirect transmission probabilities as a product of probabilities of sub-processes. Especially the way in which the effect of intervention measures are represented in (mathematical) models needs to be considered carefully. Interestingly, the work in chapter 5 provides a mechanistic illustration of how important the representation of this effect may be: if the acidification of drinking water enhances the decay of the pathogen in a certain area around the sender, it would need to be modelled as part of the environmental stage and not as a reduction at the source. Future work will have to show if such a description can explain the observed interaction effect.

The models that are developed as part of this thesis have provided important insight in the process of indirect transmission. The results of the modelling showed that the environment surrounding a source can remain contaminated for a prolonged period after a source has been emitting and thus that transmission can occur (long) after a source has been removed. This is important during an epidemic because it implies that only removing an infectious source is not enough to stop transmission and care should also be taken to avoid spread from the surrounding environment. In order to reduce disease spread by indirect transmission it is recommended to thoroughly clean the environment surrounding both an infectious source and a susceptible receiver, even after this source has been removed. An example of this is given in chapter 5 where a prediction of the model is tested and shown to be accurate in a hospital intensive care unit setting as mentioned before in the summary of the findings. This finding, that delayed transmission can be stopped by intensified cleaning, strongly emphasises the need and importance of evacuation, cleaning and disinfection measures. It is thus recommended in all situations (between-pen transmission, between-farm transmission, but also healthcare settings such as hospital wards, nursing homes, and many others) where indirect transmission of diseases is of importance that the environment and surfaces are thoroughly cleaned on a regular basis to counteract diffusive delayed transmission. Moreover, we now have the tools to determine where (in which situations) measures like intensified cleaning are necessary and how much effort is needed (i.e. in terms of frequency of cleaning).

Although to this date little is known about specific routes of indirect transmission it is important to realise that even after a source of infectious material has been taken away, the environment can still be contaminated and slow, but progressive, diffusion could cause transmission long after the removal of a source. For example in the survival experiment described in chapter 5 *E. coli* was found to survive well over 100 days in the environment. Thorough cleaning of the environment, if necessary, will thus help to decrease this form of transmission.

Extrapolating the indirect transmission model to different datasets

The indirect transmission experiments carried out in this thesis are all designed with a (relative) small distance between senders and receivers and the models are developed and fitted to these data. This is of course not the only situation indirect transmission is of importance. Whenever animals share a (confined) space there is also opportunity for indirect transmission to take place. Also there is no direct limitation to use the models described in this thesis for larger distances. An example of this is the study by Ssematimba *et al*, where the 2D diffusion model was used to have an improved estimate of the kernel for indirect transmission of Avian Influenza during the 2003 outbreak in The Netherlands [26]. The authors found a much larger value for the transmission constant D (22.7 km^2/day vs. 0.003 m^2/day), which is to be expected, because this was outside (high wind speeds) whereas the experiments were carried out inside (low wind speeds), using strict hygiene protocols and limited contact possibilities. But it does show that the diffusion model is very well adaptable to other situations.

Implications for existing knowledge / literature

The results of the experiments carried out in chapter 4 and chapter 5 show a delay of several days before the first transmission event occurs, followed by a clustering of cases after this first event. This is best described by assuming progressive (but slow) contamination of the environment surrounding the source through a diffusion process and taking into account that a pathogen decays while it diffuses through the environment. This shows that there is an explicit need to include the travel time of the pathogen in the environment in a model to describe indirect transmission over a short distance accurately. Furthermore, this also shows that the estimations for β in chapter 2 are not correct for indirect transmission. However the effects of acidification of the drinking water on indirect transmission remain unimpaired.
Indirect transmission has been studied experimentally before in a number of studies [27-33]. In some of these studies (travel) time has been taken into account in the analyses explicitly, but in others not. Given the findings in this thesis, it is interesting to reinterpret the results of these last studies keeping in mind these new findings. For example in the study by Van Roermund *et al* [28] their results show that transmission between spatially separated pens does not occur in their experimental setup. However as the authors already have mentioned there were boards along the pen sides that prevented infectious material to spread from the centre cage, this could act as a barrier for infectious material, thereby lowering the value of the diffusion constant D. Furthermore, the experiments lasted only 18 days post inoculation. From the experiments performed in chapter 5 we gained the new knowledge that, for *C. jejuni* with 5 inoculated animals in the centre cage it took 24 days before the first transmission events

occurred. Estimates for the decay rate of FMDV in the environment are highly dependent (among other factors) on temperature [34, 35]. However in conditions comparable to the conditions in our experiments the decay rate of FMDV is quite similar to *C. jejuni* [34]. It is thus possible that indirect transmission of FMDV does occur between spatially separated pens but that this process is slower than the authors assumed in their study. Another example is the study by Klinkenberg *et al* [27] in which the authors describe a new method to calculate the basic reproduction ratio for transmission experiments in which for both direct and indirect transmission the transmission parameter was estimated. Given the new insight obtained in this thesis these calculations should be repeated with a more appropriate model for the between-pen transmission.

A similar line of reasoning holds for the study of Dekker *et al* [32] where the indirect transmission of *Streptococcus suis* between spatially separated pigs is modelled as a build-up in the environment, without a spatial component or taking into account that there is decay of the pathogen in the environment. As such, this model would not be able to explain the difference in delay observed in this thesis.

In their study Charleston *et al* [36] argued that for example for FMDV in cattle (responsible for huge economic losses worldwide [37]) there is no transmission before onset of clinical symptoms; thereby the authors imply that pre-emptive control measures may be unnecessary and instead more effort should made in early detection of infection followed by a fast intervention. However, in their study the authors placed infectious animals together with susceptible animals in a clean stable (clean environment) for only eight hours and they thus estimated only the effect of direct transmission. The results in this thesis however show that indirect transmission is also of major importance and the effects of indirect transmission should be taken into consideration. When animals are infectious they will shed virus into the environment, thus contaminating the environment. Other animals in the same environment will now be exposed to a combined force of infection from both direct transmission and indirect transmission even after the infected animals are removed. This means that culling of the animals is still a necessity and on top of that the environment should be thoroughly cleaned/ disinfected to prevent indirect transmission to cause further infections.

Lastly in the study of Warfel *et al* [33], the authors claim that the transmission of *Bordetella pertussis* between spatially separated baboons occurs via aerosolised respiratory droplets. Again, with the current knowledge it is more likely that the transmission is via slow contamination of the environment through a diffusive process and that there is build-up of *Bordetella pertussis* in the environment, causing the delay of 19 days the authors report in their study before first transmission events occur between the spatially separated animals.

Future work

The work presented in this thesis is only a start in the search for underlying mechanisms of indirect transmission. Most of the studies carried out are based on data acquired from carefully controlled experiments. One thing that is not thoroughly tested in this thesis is the transmission over longer distances. The 2D-diffusion model does predict there is a limit to the distance a pathogen can spread in high enough quantities to cause transmission, if there

is decay of the pathogen in the environment. The exact distance that can be reached is dependent on both the decay and the diffusion constant D. This is a clear and straightforward to test prediction and can be used to further explore the generalisability of the model. The next step in research should focus on long(er) distance spread in less controlled field conditions. It should be noted however, that carrying out experiments in the field is extremely difficult and requires careful planning and interpretation. One way to overcome this issue is to make use of data from previous outbreaks if that data is of suitable quality. When developing contingency plans, researchers could be involved to discuss how sampling and data collection can be addressed whilst combating the outbreak. This would be of tremendous value for future research.

Field experiments focussing on the dispersion of infectious material in the environment surrounding a source can contribute valuable information about the dispersion process under non-controlled circumstances. A possibility to study indirect transmission in more detail would be to make use of an attenuated vaccine to track the spread of the attenuated virus in the population and in the environment. Sampling of these attenuated vaccine particles in the environment can be done without the need to perform experiments in confined spaces. Furthermore bio-security measures that clean the environment should be tested for their efficacy, again, if possible, under field conditions. Chapter 5 in this thesis already has shown that an intensified cleaning routine can stop the indirect transmission component in a hospital intensive care unit setting.

Lastly, the current assumptions about transmission between spatially separated hosts (or temporally separated in the same space) should be revisited keeping this recently gained knowledge in mind. As is shown, transmission of pathogens is not always instantaneous. There is time needed for the infectious material to get from a source to a receiver. There is thus a need to track infectious individuals in time and space up until the time of interest.

Overall Conclusions

- Acidification of the drinking water reduces indirect transmission but not direct transmission of *C. jejuni* between broilers and can be used as a modulation factor to study indirect transmission.
- Acidification of the drinking water has an effect on indirect transmission of *C. jejuni* both when applied to the sender side of the indirect transmission process and when applied to the receiver side of the process.
- Unless the pathogens spends a negligible period of time in the environment between sender and receiving animal, explicit inclusion of the period in the environment in a transmission model is needed to obtain an accurate description of the transmission process. In particular a model of two-dimensional diffusive transfer is able to explain the experimental observations in this thesis, as well as observations on the spread of Vancomycin-Resistant *Enterococcus* in an intensive care unit.
- The difference between *C. jejuni* and *E. coli* in terms of the observed delay until occurrence of the first infection events can quantitatively be explained from the differences in decay rates of these bacteria in the environment.

- Transmission of pathogens with low decay rates can occur at distant locations long after the source of infectious material is removed.
- To extrapolate to the low doses associated with indirect transmission, a biologically correct model, one that abides the independent action hypothesis is needed.

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Summary

The central motivation for the research described in this thesis are the disease transmission events that occurred during the last major epidemics of livestock diseases (e.g. Foot and Mouth Disease Virus (FMDV) and Classical Swine Fever Virus (CSFV)) in The Netherlands and abroad, despite having movement bans in place and other (bio-)security measures. The occurrence of these transmission events although existing movement bans where in place imply that there was unreported illegal transport (which is not very likely, but might explain a small fraction of the events) and/or that the pathogen is spread by indirect transmission (transmission without direct host-host contact). This last form of transmission is often termed “neighbourhood transmission” and it is estimated that this form of transmission was responsible for the majority of the infections, for example for the recent major outbreak of CSFV in 1997/1998 in The Netherlands estimations range from 60-80%. This neighbourhood transmission most probably consists of indirect transmission, because there is no direct contact between animals on the different farms when transmission occurs.
To combat and control an outbreak of a highly transmissible livestock disease different instruments are available, for example, culling of animals, vaccination of animals and different bio-security measures such as a ban on all transport of animals and animal products during an outbreak, implementation of hygiene protocols for visitors on and off the farms etc. Because these major outbreaks have enormous socio-economic effects due to bans on livestock movements, animal culling, trade standstills and export bans (the costs of the FMD outbreak in the United Kingdom in 2001 were estimated to be about €4.1 billion) a better understanding of indirect transmission is necessary to put in place evidence based bio-security measures against (indirect) neighbourhood transmission. Therefore, the goal of this thesis was to gain more insight in the underlying mechanisms of transmission between spatially separated hosts (i.e. indirect transmission).

Indirect transmission can be thought of as a process consisting of three separate sub-processes: 1) an infectious sender that excretes infectious material; 2) traveling of the infectious material through the environment outside the host, getting from sender to receiver via some route; and 3) a susceptible receiver that takes in (some) of the infectious material and is getting infected by this material. To study the mechanisms of indirect transmission in more detail a factor to modulate transmission was used that could potentially help to gain insight in the separate sub-processes of indirect transmission. In broiler chickens the use of fermented liquid feed has been shown to reduce the susceptibility to *Campylobacter jejuni* (*C. jejuni*) and *Salmonella*. It is thus possible that acidification of the drinking water could be used as a modulation factor for transmission. Therefore the effect of acidification of the drinking water on the transmission parameters of direct and indirect transmission was studied (chapter 2). The results of this study showed that acidified drinking water reduces indirect transmission but not direct transmission of *C. jejuni* between broilers. Furthermore, it showed that, in terms of the transmission parameter β, there is a difference between direct and indirect transmission, with the estimated value for β for indirect transmission being two orders of magnitude lower than for direct transmission ($\beta = 3.7$ day^{-1} for direct transmission versus $\beta = 0.011$ day^{-1} for indirect transmission). Thus, indirect transmission occurs at a lower rate than direct transmission.

The exact underlying causes of the effect of acidification of the drinking water on indirect transmission are not clear. The effect can (theoretically) be sorted in any of the sub-processes of indirect transmission. I.e. the effect could be associated with the sending host, it could be that the effect occurs during the travelling from sender to receiver or it could take place at the receiving host. A combination of effects at the different sub-processes is also possible. To get more insight in where exactly this effect takes place the sender and receiver sub-process were studied in more detail (chapter 3). To this end an indirect transmission experiment was carried out where either the sender animals received acidified drinking water, or receiving animals received acidified drinking water, or both sender and receiving animals received acidified drinking water. The results showed that a significant negative interaction effect between acidification of the sender and receiver sub-processes exists. Quantitatively the results indicate that there is no additional effect of acidification of the drinking water of both sides of the transmission process compared to acidified drinking water of only one side.

To study the transport of the pathogen in the environment in more detail, a series of novel, tailor-made indirect transmission experiments was carried out. The results from the transmission experiments showed a large delay before the first transmission event and a clustering of transmission events subsequently taking place. Existing transmission models (SIR-framework) cannot explain these results in a satisfactory way. The existing models do allow for a large delay before the first infection event by having a low value for the transmission parameter β, but are unable to explain the clustering of the subsequent transmission events, because such a low value for β would imply that subsequent transmission events would also have large delays between them. Thus, to have a better, mechanistic explanation for the patterns found in the transmission experiment, a novel model framework was developed to study indirect transmission between spatially separated hosts. This model framework is setup as a compartmental model in which the time that the pathogen has spent in the environment outside the host is explicitly taken into account by one or more separate environmental reservoirs. Using this model framework it was found that the observed transmission pattern is best described by a multistage environmental route from the sending animals to the receiving animals, which suggests that indirect transmission occurs through progressive (but slow) contamination of the environment surrounding the source (chapter 4). The best fitting, resulting model also identifies the decay rate of a pathogen in the environment outside the host as a key parameter. Based on this, one of the predictions of this model is that the observed delay in onset of transmission is dependent of the decay rate of the pathogen. To formulate this more precisely: the model predicts that the faster a pathogen decays in the environment the longer the delay before onset of first transmission will be.

To test the prediction that the delay before onset of transmission is dependent on the decay rate of the pathogen the indirect transmission experiments where repeated with both *C. jejuni* and (a marked) *Escherichia coli* (*E. coli*) in chapter 5. In these experiments broiler chickens where inoculated with only *C. jejuni* or with both *C. jejuni* and *E. coli*. The results showed that for *C. jejuni* it takes much longer for the first effective bacterium to cross the small distance of approximately 75 cm than it does for *E. coli*. As both pathogens are excreted in similar amounts and are transported on the same material (feaces) any difference in delay before

onset of transmission are attributable to the difference in decay rate of the two pathogens in the environment. A general model was developed, capable of explaining the observed patterns from both pathogens. Not only does this model explicitly take into account the distance between hosts; it also takes into account the amount of time a pathogen spends (travels) in the environment. During this travel time the pathogen is decaying. The resulting model describes the spread of infectious material in the environment with the aid of a two-dimensional diffusion model. Predictions of this model were tested using data of Vancomycin-Resistant *Enterococci* in a hospital intensive care unit. The model predicts a delayed transmission component and indeed the existence of this component is shown in such a setting; when a thorough cleaning regime is applied, this delayed component is not observed anymore and thus the transmission risk is reduced.

The model in chapter 5 explains the delayed transmission from a delayed increase in pathogen concentration at the distant location/cage. Based on a plausible dose-response assumption the model predicts that this delayed increase in concentration leads to a period with “zero” infection rate followed by a sharp onset of a period with non-zero rate of infection.

The dose-response assumption of the model of chapter 5 is that the infection hazard of the recipient host is proportional to the pathogen concentration in its location/cage. It can be shown that this assumption, if one also assumes that the rate at which pathogens are entering the host is proportional to the local concentration in the environment, corresponds to assuming an exponential dose-response relationship. This exponential relationship is equivalent to assuming that each single bacterium entering the host represents an independent probability of infection of the host, and that this probability is the same for each individual bacterium. In chapter 6 the form of the dose-response curve is studied in detail for *C. jejuni* by carrying out a dose-response experiment. A range of dose-response models were compared and tested how well these fitted to the data from the dose-response experiment. Here it was shown that for interpolating purposes two relatively simple models are best capable of describing the data from the dose-response experiment. However these models do not abide the independent action hypothesis that each pathogen particle acts independently of other particles. For extrapolating purposes it was shown that from the models that were studied a hypergeometric dose-response model is the best fitting model. This model does abide the independent action hypothesis and is thus biologically correct for low doses.

To summarise, the research described in this thesis has provided valuable insights in the mechanisms that underlie indirect transmission. It has shown that standard transmission models are unable to explain indirect transmission observed in controlled experiments, and that a good description can be achieved after explicit inclusion of a suitable model for the period spent by the pathogen in the environment between sender and receiving individual. In particular a model of two-dimensional diffusive transfer is able to explain the experimental observations, as well as observations on the spread of Vancomycin-Resistant *Enterococcus* in an intensive care unit.

Main conclusions of this thesis:

- Acidification of the drinking water reduces indirect transmission but not direct transmission of C. jejuni between broilers and can be used as a modulation factor to study indirect transmission.
- Acidification of the drinking water has an effect on indirect transmission of C. jejuni both when applied to the sender side of the indirect transmission process and when applied to the receiver side of the process.
- Unless the pathogens spends a negligible period of time in the environment between sender and receiving animal, explicit inclusion of the period in the environment in a transmission model is needed to obtain an accurate description of the transmission process. In particular a model of two-dimensional diffusive transfer is able to explain the experimental observations in this thesis, as well as observations on the spread of Vancomycin-Resistant Enterococcus in an intensive care unit.
- The difference between C. jejuni and E. coli in terms of the observed delay until occurrence of the first infection events can quantitatively be explained from the differences in decay rates of these bacteria in the environment.
- Transmission of pathogens with low decay rates can occur at distant locations long after the source of infectious material is removed.
- To extrapolate to the low doses associated with indirect transmission, a biologically correct model, one that abides the independent action hypothesis is needed.

Samenvatting

De belangrijkste motivatie voor het onderzoek beschreven in dit proefschrift is de verspreiding van virussen tijdens de laatste (grote) epidemieën van dierziekten (i.e.mond-en-klauwzeer (MKZ), Hoogpathogene Vogelgriep (HPAI) en klassieke varkenspest (KVP)) in Nederland en daarbuiten, ondanks een volledig vervoersverbod voor vee en andere (bio-) veiligheidsmaatregelen. Het feit dat virusverspreiding plaatsvindt ondanks de ingestelde vervoersverboden impliceert dat er of illegaal transport plaats heeft gevonden (wat niet heel erg aannemelijk is, maar het zou een kleine fractie van de gebeurtenissen kunnen verklaren) en/of dat de ziekteverwekker verspreid is door middel van indirecte transmissie (transmissie zonder direct contact). Deze laatste vorm van transmissie wordt vaak “buurt-transmissie” genoemd en er wordt ingeschat dat deze vorm van transmissie verantwoordelijk is voor het leeuwendeel van de infecties. De schattingen voor het aandeel van buurt-transmissie voor de recente epidemie van KVP in Nederland in de periode 1997/1998 liggen tussen de zestig en tachtig procent. Deze buurt-transmissie bestaat hoogstwaarschijnlijk uit indirecte transmissie aangezien er geen direct contact tussen de dieren op de verschillende boerderijen is ten tijde van de transmissie.

Voor de bestrijding en controle van zeer besmettelijke dierziekten zijn een aantal instrumenten beschikbaar, zoals het ruimen (van dieren), het toepassen van vaccinatie en verschillende bioveiligheidsmaatregelen. Voorbeelden van deze bioveiligheidsmaatregelen zijn een vervoersverbod voor dieren en dierproducten tijdens een uitbraak en implementatie van strikte hygiëneprotocollen voor bezoekers van veehouderijen. Deze epidemieën hebben grote sociaal-economische gevolgen door het vervoersverbod voor vee, ruiming van de dieren, handelsstilstand en exportverboden (de kosten voor de MKZ uitbraak in 2001 in Groot-Brittannië zijn geschat op €4.1 miljard). Deze sociaal-economische gevolgen pleiten voor een beter begrip van indirecte transmissie om empirisch onderbouwde bioveiligheidsmaatregelen tegen (indirecte) buurt-transmissie te ontwikkelen en te implementeren. Het doel van dit proefschrift was daarom om meer inzicht te verkrijgen in de onderliggende mechanismen van transmissie tussen ruimtelijk gescheiden gastheren (d.w.z. indirecte transmissie).

Indirecte transmissie kan beschouwd worden als een proces dat is op te splitsen in drie subprocessen: 1) een infectieuze zender, welke infectieus materiaal uitscheidt; 2) transport van infectieus materiaal door de omgeving van zender naar ontvanger via een bepaalde route; en 3) een vatbare ontvanger die (een gedeelte) van het infectieus materiaal inneemt en geinfecteerd raakt door dit infectieus materiaal. Om de mechanismen van indirecte transmissie in meer detail te bestuderen is er gebruik gemaakt van een factor die mogelijk indirecte transmissie kan beinvloeden (verminderen), iets dat mogelijk kan leiden tot een beter inzicht in de afzonderlijke subprocessen van indirecte transmissie. In vleeskuikens is aangetoond dat het gebruik van gefermenteerd vloeibaar voedsel leidt tot verminderde vatbaarheid voor *Campylobacter jejuni* (*C. jejuni*) en *Salmonella*. Mogelijk kan aanzuring van het drinkwater daarom gebruikt worden als modulatiefactor voor indirecte transmissie. Het effect van aanzuring van drinkwater op de transmissieparameters voor directe en indirecte transmissie is daarom bestudeerd in hoofdstuk 2. De resultaten van deze studie lieten zien dat aanzuring van het drinkwater een reducerend effect heeft op indirecte transmissie van *C. jejuni* tussen vleeskuikens, maar niet op directe transmissie. Bovendien werd gevonden dat

de transmissie parameter, β, verschilt tussen directe en indirecte transmissie; de geschatte waarde voor β voor indirecte transmissie (β=0.011 dag^{-1}) is twee ordes van grootte lager dan voor directe transmissie (β=3.7 dag^{-1}). Indirecte transmissie vindt dus plaats met een verminderde verspreiding vergeleken met directe transmissie.

De precieze onderliggende oorzaken van het effect van aanzuren van drinkwater op indirecte transmissie zijn niet duidelijk. Dit effect kan (theoretisch) worden veroorzaakt in elk van de subprocessen van indirecte transmissie. Dat wil zeggen het effect kan geassocieerd zijn met het infectieuze zenderdier, het kan zijn dat het effect ontstaat gedurende het transport van zender naar ontvanger of dat het plaatsvindt bij de ontvangende gastheer. Ook is een combinatie van effecten in de verschillende subprocessen mogelijk. Om meer inzicht te krijgen in waar dit effect precies wordt gesorteerd werd het zender- en ontvangersubproces nader bestudeerd (hoofdstuk 3). Hiervoor werd een indirect transmissieexperiment uitgevoerd waar, of de zenderdieren aangezuurd drinkwater aangeboden kregen, of de ontvangerdieren, of zowel de zender- als ontvangerdieren. De resultaten van dit experiment lieten zien dat er een significant negatief interactie effect bestaat tussen het aanzuren van drinkwater van de zenderdieren en het aanzuren van het drinkwater van de ontvangerdieren. Kwantitatief betekent dit dat er geen additioneel effect is van het aanzuren van drinkwater aan beide kanten van het transmissieproces vergeleken met het aanzuren van het drinkwater aan één kant van het transmissieproces.

Om subproces 2, het transport van infectieus materiaal door de omgeving van zender- naar ontvangerdier nader te bestuderen werden er een reeks, vernieuwende, op maat gemaakte, indirecte transmissie experimenten uitgevoerd. De resultaten van deze transmissieexperimenten lieten een grote vertraging zien voordat de eerste transmissie gebeurtenissen optraden en dat hierna een clustering van transmissiegebeurtenissen optreedt. Deze bevindingen kunnen niet op een bevredigende manier worden beschreven door reeds bestaande modellen (SIR-raamwerk). Hoewel de vertraging tot de eerste transmissiegebeurtenis in principe wel beschreven kan worden door de bestaande modellen door een lagere waarde voor de transmissieparameter β aan te nemen, kan de daaropvolgende clustering van transmissiegevallen niet goed worden beschreven door een lagere waarde voor β, aangezien een lage waarde impliceert dat daaropvolgende transmissie gevallen ook een grote vertraging tussen elkaar zouden moeten vertonen. Om een betere, mechanistische verklaring te geven voor de patronen zoals geobserveerd in de transmissieexperimenten is een nieuw model-raamwerk ontwikkeld waarin de tijd die een pathogeen doorbrengt in de omgeving buiten de gastheer expliciet is opgenomen door middel van één of meerdere omgevingsreservoirs. Door gebruik te maken van dit model-raamwerk bleek dat het geobserveerde patroon het best beschreven kan worden door een model bestaande uit een route via meerdere geschakelde omgevingsreservoirs van zender- naar ontvangerdieren. Dit suggereert dat indirecte transmissie plaatsvindt door (langzaam) voortgaande besmetting van de omgeving rondom de bron (hoofdstuk 4). Verder bleek het best passende model de sterfte van het pathogeen in de omgeving als (één van de) belangrijkste parameter aan te geven. Hieruit vloeit één voorspelling van het model voort: de geobserveerde vertraging tot de eerst transmissiegebeurtenis is afhankelijk van de sterfteparametervan het pathogeen in de

omgeving. Preciezer geformuleerd: het model voorspelt dat als pathogenen sneller afsterven in de omgeving het langer duurt tot de eerste transmissiegebeurtenis plaatsvindt.
Om deze modelvoorspelling te toetsen zijn de indirecte transmissie experimenten herhaald met zowel *C. jejuni* als (een gemarkeerde) *Escherichia coli* (*E. coli*) in hoofdstuk 5. In deze experimenten werden vleeskuikens geinoculeerd met alleen *C. jejuni* of met de combinatie van *C. jejuni* en *E. coli*. De resultaten van deze experimenten lieten zien dat het voor *C. jejuni* veel meer tijd kost om de korte afstand van circa 75 cm van zender- naar ontvangerdieren te overbruggen dan de tijd die het *E. coli* kost om dezelfde afstand te overbruggen. Aangezien beide pathogenen in dezelfde hoeveelheden worden uitgescheiden en op hetzelfde (faecale) materiaal worden getransporteerd is het verschil in tijd tot eerste transmissiegebeurtenis toe te schrijven aan het verschil in overlevingstijd (1/sterfteparameter) in de omgeving buiten de gastheer. Om de verschillen in geobserveerde patronen te verklaren werd een nieuw model ontwikkeld dat niet alleen expliciet de afstand tussen gastheren in acht neemt maar ook de tijd dat een pathogeen verblijft (reist) in de omgeving buiten de gastheer. Gedurende deze reistijd vindt sterfte van de pathogeen plaats. Het resulterende model beschrijft de verspreiding van infectieus materiaal in de ruimte (omgeving) door middel van een tweedimensionaal diffusiemodel. Predicties van dit model werden getest door gebruik te maken van data van Vancomycine-resistente *Enterococcus* in een intensivecare-afdeling van een ziekenhuis. Het model voorspelt een vertraagde transmissiecomponent en het is aangetoond dat deze component voorkomt in een dergelijke omgeving. Wanneer er een grondig schoonmaakregime wordt toegepast verdwijnt deze vertraagde component waardoor het risico van verspreiding wordt gereduceerd. Het model zoals beschreven in hoofdstuk 5 geeft een verklaring voor de vertraagde transmissie doordat er een vertraagde toename van pathogeen concentratie is in de op afstand gelegen ontvangerkooi. Door een plausibele dosis-responsaanname te doen voorspelt het model dat deze vertraagde toename in concentratie leidt tot een periode waarin de infectiesnelheid (zo goed als) nul is, gevolgd door een scherpe overgang naar een periode waarin de infectiesnelheid groter dan nul is.
De dosis-responsaanname in het model van hoofdstuk 5 is dat het infectierisico voor de ontvangende gastheer evenredig is met de pathogeenconcentratie op die plaats. Er kan worden aangetoond dat deze aanname, als ook wordt aangenomen dat de snelheid waarmee pathogenen door de gastheer worden opgenomen evenredig is met de lokale concentratie in de omgeving, correspondeert met een exponentiele dosis-responsrelatie. Deze exponentiële dosis-responsrelatie is equivalent aan de aanname dat iedere afzonderlijke bacterie die in de gastheer komt een onfhankelijke kans heeft om tot infectie te leiden en dat deze kans gelijk is voor elke individuele bacterie. In hoofdstuk 6 is de vorm van de dosis-reponsecurve voor *C. jejuni* in vleeskuikens in detail bestudeerd door middel van dosis-responsexperimenten. Verschillende dosis-responsmodellen werden vergeleken en getest werd hoe goed deze passen bij data verkregen uit de dosis-responsexperimenten. Dit hoofdstuk laat zien dat voor interpolatiedoeleinden twee relatief simpele modellen het beste zijn in het beschrijven van de data van de dosis-reponsexperimenten. Echter houden deze modellen zich niet aan de zogenaamde independent-actionhypothese dat ieder pathogeendeeltje onafhankelijk van andere deeltjes handelt.Voor extrapolatiedoeleinden is aangetoond dat, van de modellen die

zijn bestudeerd, een hypergeometrisch dosis-responsmodel het meest passende model is. Dit model houdt zich wel aan de independent-actionhypothese en is dus ook voor lage dosis biologisch correct.
Het onderzoek beschreven in dit proefschrift heeft waardevolle inzichten opgeleverd in de onderliggende mechanismen van indirecte transmissie. Verder is gebleken dat de standaard transmissiemodellen niet in staat zijn om een goede verklaring te geven voor de indirecte transmissie zoals deze geobserveerd is in gecontroleerde experimenten. Een goede verklaring kan wel worden gegeven door expliciet de tijd die een pathogeen doorbrengt in de omgeving tussen zender- en ontvangerdieren op te nemen in een model. Met name een model dat uitgaat van tweedimensionale diffuse verplaatsing van pathogeendeeltjes is in staat zowel de experimentele observaties correct te beschrijven als ook de verspreiding van Vancomycine-resistente *Enterococcus* in een intensivecare-afdeling.

Conclusies van dit proefschrift:

- Aanzuren van het drinkwater reduceert indirecte transmissie maar niet directe transmissie van *C. jejuni* tussen vleeskuiken en kan worden gebruikt als modulatiefactor om indirecte transmissie te bestuderen.
- Aanzuring van het drinkwater heeft een effect op indirecte transmissie zowel als het wordt toegepast op de zenderkant van het indirecte transmissieproces als op de ontvangerkant van het transmissieproces.
- Het is noodzakelijk om de periode doorgebracht in de omgeving expliciet op te nemen in een transmissiemodel voor een accurate beschrijving van het transmissieproces, tenzij het pathogeen een te verwaarlozen tijd doorbrengt in de omgeving tussen zender- en ontvangerdier. In het bijzonder een model dat uitgaat van tweedimensionale diffuse verplaatsing van pathogenen is in staat om zowel de experimentele observaties correct te beschrijven als de verspreiding van Vancomycine-resistente *Enterococcus* in een intensivecare-afdeling.
- Het verschil tussen *C. jejuni* en *E. coli* met betrekking tot de geobserveerde vertraging tot het optreden van de eerste transmissiegebeurtenis kan kwantitatief worden uitgelegd aan de hand van de verschillen in sterfteparameters tussen deze twee bacteriën.
- Transmissie van een pathogeen met lage sterfte kan plaatsvinden op locaties ververwijderd van een bron, reeds lang nadat de bron van het infectieuze materiaal is verdwenen.

Dankwoord

En dan mag ik eindelijk het laatste stuk schrijven van mijn proefschrift…
Dan denk je na vier jaar (en een beetje) dat je “het” wel even afrondt naast je nieuwe baan in een ander land… Goed, dat duurde iets langer dan gehoopt, maar het eindresultaat is er dan toch! En hoewel mijn naam op de voorkant staat, weet ik zeker dat ik het hele traject nooit op deze manier zou hebben doorlopen zonder de steun / kennis / vriendschap / geduld van veel mensen!

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About the author

Curriculum Vitae

Bram Alexander Daniël van Bunnik was born on August 3th 1978 in Almelo. In 1996 he graduated from the "St. Canisius" in Almelo, after which he started studying Computer Science at the University of Groningen. After one year, however, he decided this was not the study for him and instead started studying Biology at the University of Groningen in 1997. He graduated in August 2006. As of September 2007 he started his PhD at the Department of Quantitative Veterinary Epidemiology of Wageningen University and was stationed throughout his PhD at the department of Epidemiology and Crisis organisation and Diagnostics of the Central Veterinary Institute part of Wageningen UR in Lelystad. His project involved combining emperical data with mathematical modelling to seek mechanisms that underly indirect transmission between spatially seperated animals. At present, Bram is working at the Epidemiology Research Group, of the University of Edinburgh, Scotland as a post-doctoral researcher. His project involves studying the flow of antibiotic resistance genes between different reservoirs using mathematical models. He is particular interested in the spread of methicillin resistant *Staphylococcus aureus* between hospitals and other settings.

List of publications

Refereed scientific journals

van Bunnik BAD, Ssematimba A, Hagenaars TJ, Nodelijk G, Haverkate MR, Bonten MJM, Hayden MK, Weinstein RA, Bootsma MCJ, De Jong MCM: Small distances can keep bacteria at bay for days. Proceedings of the National Academy of Sciences 2014, 111(9):3556-3560.

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Conference proceedings and abstracts

van Bunnik BAD, Ssematimba A, Hagenaars TJ, Nodelijk G, Haverkate MR, Bonten MJM, Hayden MK, Weinstein RA, Bootsma MCJ, De Jong MCM: Small distances can keep bacteria at bar for days. ISVEE Conference, 2012, Maastricht, The Netherlands. Oral presentation.

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van Bunnik BAD, Hagenaars TJ, Katsma WEA, Bolder NM, Nodelijk G, de Jong MCM: Unexpected Delay in Transmission between Spatially Separated Hosts. InFER, 2011, Warwick, United Kingdom. Poster presentation.

van Bunnik BAD, Hagenaars TJ, Katsma WEA, Bolder NM, Nodelijk G, de Jong MCM:Unexpected Delay in Transmission between Spatially Separated Hosts. SVEPM, 2011, Leipzig, Germany. Poster presentation.

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van Bunnik BAD, Hagenaars TJ, Katsma WEA, Bolder NM, Nodelijk G, de Jong MCM: Modelling indirect transmission of Campylobacter in broilers. VEEC, Deventer, 2009, The Netherlands. Oral presentation.

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Training and Supervision Plan

Training and Supervision Plan Wageningen Institute of Animal Sciences (WIAS)	
Education and Training	**Year**
The basic package (3 ECTS)	
WIAS Introduction Course	2008
Course on philosophy of science and/or ethics	2009
Scientific Exposure (18 ECTS)	
International conferences	
International Symposium on Veterinary Epidemiology and Economics (VEEC)	2009
Annual meeting of the Society for Veterinary Epidemiology and Preventive Medicine (SVEPM)	2009
International Symposium on Veterinary Epidemiology and Economics (ISVEE), 2009	2009
Annual meeting of the Society for Veterinary Epidemiology and Preventive Medicine (SVEPM)	2011
International Symposium on Veterinary Epidemiology and Economics (ISVEE), 2012	2012
Seminars and workshops	
Communicatie Workshop Integrale Analyse Dierziekten, Den Haag, 2007	2007
WIAS Science Day, Wageningen, 2008	2008
Workshop Horizontale Oplossingrichtingen, Lelystad, 2008	2008
Campylobacter Symposium, Amersfoort, The Netherlands, 2008	2008
CVI onderzoeksdag 2009/2010	2009
WIAS Science Day, Wageningen, 2009	2009
Annual meeting Dutch Society of Theoretical Biology, Schoorl, The Netherlands, 2009	2009
Workshop Jong ASG - Jong LEI Ethiek in onderzoek en beleid, Den Haag, The Netherlands	2009
Epizone 5th Annual meeting, Arnhem, The Netherlands	2011
WIAS Science Day, Wageningen, 2011	2011
Reviewing a Scientific Paper (WGS workshop) , Wageningen, 2011	2011
Presentations	
Oral presentations at monthly scientific meeting QVERA/QVE	2007-2011
Poster presentation at WIAS Science Day, 2008	2008
Oral presentation Workshop Horizontale Oplossingsrichtingen, Lelystad, 2008	2008
Oral presentation WIAS Science Day, 2009	2009

Oral presentation at the International Symposium on Veterinary Epidemiology and Economics (ISVEE), 2009	2009
Poster presentation at WIAS Science Day 2010	2010
Poster presentation at the Epizone meeting 2011	2011
Poster presentation at WIAS Science Day 2011	2011
Oral presentation at the International Symposium on Veterinary Epidemiology and Economics (ISVEE), 2012	2012
In-Depth Studies (14 ECTS)	
Disciplinary and interdisciplinary courses	
Orientation on Mathematical Modelling in Biology, WIAS, Wageningen	2008
Epidemiology and Control of Infectious Disease, Imperial College London, London, United Kingdom	2010
Advanced statistics courses	
Advanced statistics for the life sciences, WIAS, Wageningen	2011
PhD students' discussion groups	
Monthly scientific meeting QVERA/QVE	2007-2011
Reading group on mathematical modelling of infectious disease	2008-2009
Journal Club QVERA/QVE	2010-2011
Statutory Courses (3 ECTS)	
Use of Laboratory Animals, Faculteit Diergeneeskunde, Utrecht	2011
Professional Skills Support Courses (3 ECTS)	
Writing for academic publications, Lelystad	2008
High Impact Writing in Science, WGS, Wageningen	2012
Research Skills Training (2 ECTS)	
Preparing own PhD research proposal	2007
Didactic Skills Training (2 ECTS)	
Lecturing	
MTEC Course	2009
Education and Training Total	**45 ECTS**

Colophon

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